

VISIT...

LANZAROTE
Caliente.COM

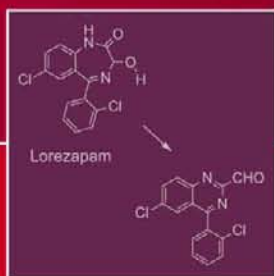
DRUGS AND THE PHARMACEUTICAL SCIENCES

VOLUME 210

PHARMACEUTICAL STRESS TESTING

PREDICTING DRUG DEGRADATION

SECOND EDITION



Steven W. Baertschi
Karen M. Alsante
Robert A. Reed

informa
healthcare

Pharmaceutical Stress Testing

DRUGS AND THE PHARMACEUTICAL SCIENCES

Series Executive Editor

James Swarbrick
PharmaceuTech, Inc.
Pinehurst, North Carolina, USA

Advisory Board

Larry L. Augsburger
University of Maryland
Baltimore, Maryland,
USA

Robert Gurny
University of Geneva
Geneva, Switzerland

Joseph W. Polli
GlaxoSmithKline
Research Triangle Park
North Carolina, USA

Elizabeth M. Topp
Purdue University
West Lafayette, Indiana, USA

Harry G. Brittain
Center for Pharmaceutical
Physics
Milford, New Jersey, USA

Anthony J. Hickey
University of North Carolina
School of Pharmacy
Chapel Hill, North Carolina, USA

Kinam Park
Purdue University
West Lafayette, Indiana, USA

Geoffrey T. Tucker
University of Sheffield
Royal Hallamshire Hospital
Sheffield, UK

Jennifer B. Dressman
University of Frankfurt
Institute of Pharmaceutical
Technology, Frankfurt, Germany

Jeffrey A. Hughes
University of Florida
College of Pharmacy
Gainesville, Florida, USA

Yuichi Sugiyama
University of Tokyo
Tokyo, Japan

Peter York
University of Bradford
School of Pharmacy
Bradford, UK

Recent Titles in Series

- 209. *Pharmaceutical Process Scale-Up, Second Edition*; Michael Levin, ISBN 9781616310011, 2011
- 208. *Sterile Drug Products: Formulations, Packaging, Manufacturing and Quality*; Michael K. Akers, ISBN 9780849339966, 2010
- 207. *Advanced Aseptic Processing Technology*; James Agalloco, James Akers, ISBN 9781439825433, 2010
- 206. *Freeze-Drying/Lyophilization of Pharmaceutical & Biological Products, Third Edition*; Louis Rey, Joan May, ISBN 9781439825754, 2010
- 205. *Active Pharmaceutical Ingredients: Development, Manufacturing, and Regulation, Second Edition*; Stanley Nusim, ISBN 9781439803363, 2009
- 204. *Generic Drug Product Development: Specialty Dosage Forms*; Leon Shargel, Isadore Kanfer, ISBN 9780849377860, 2010
- 203. *Pharmaceutical Statistics: Practical and Clinical Applications, Fifth Edition*; Sanford Bolton, ISBN 9781420074222, 2009

Pharmaceutical Stress Testing

Predicting Drug Degradation

Second Edition

Edited by

Steven W. Baertschi

*Research Fellow, Analytical Sciences R&D,
Eli Lilly and Company, Indianapolis, Indiana, USA*

Karen M. Alsante

*Research Fellow, Pfizer, Inc.
Groton, Connecticut, USA*

Robert A. Reed

*Vice President, CMC and Technical Operations,
Celsion Corporation, Columbia, Maryland, USA*

informa
healthcare

First edition published in 2005 by Informa Healthcare, Telephone House, 69-77 Paul Street, London EC2A 4LQ, UK.

This edition published in 2011 by Informa Healthcare, Telephone House, 69-77 Paul Street, London EC2A 4LQ, UK.

Simultaneously published in the USA by Informa Healthcare, 52 Vanderbilt Avenue, 7th Floor, New York, NY 10017, USA.

Informa Healthcare is a trading division of Informa UK Ltd. Registered Office: 37–41 Mortimer Street, London W1T 3JH, UK. Registered in England and Wales number 1072954.

© 2011 Informa Healthcare, except as otherwise indicated
No claim to original U.S. Government works

Reprinted material is quoted with permission. Although every effort has been made to ensure that all owners of copyright material have been acknowledged in this publication, we would be glad to acknowledge in subsequent reprints or editions any omissions brought to our attention.

All rights reserved. No part of this publication may be reproduced, stored in a retrieval system, or transmitted, in any form or by any means, electronic, mechanical, photocopying, recording, or otherwise, unless with the prior written permission of the publisher or in accordance with the provisions of the Copyright, Designs and Patents Act 1988 or under the terms of any licence permitting limited copying issued by the Copyright Licensing Agency Saffron House, 6-10 Kirby Street, London EC1N 8TS UK, or the Copyright Clearance Center, Inc., 222 Rosewood Drive, Danvers, MA 01923, USA (<http://www.copyright.com/> or telephone 978-750-8400).

Product or corporate names may be trademarks or registered trademarks, and are used only for identification and explanation without intent to infringe.

This book contains information from reputable sources and although reasonable efforts have been made to publish accurate information, the publisher makes no warranties (either express or implied) as to the accuracy or fitness for a particular purpose of the information or advice contained herein. The publisher wishes to make it clear that any views or opinions expressed in this book by individual authors or contributors are their personal views and opinions and do not necessarily reflect the views/opinions of the publisher. Any information or guidance contained in this book is intended for use solely by medical professionals strictly as a supplement to the medical professional's own judgement, knowledge of the patient's medical history, relevant manufacturer's instructions and the appropriate best practice guidelines. Because of the rapid advances in medical science, any information or advice on dosages, procedures, or diagnoses should be independently verified. This book does not indicate whether a particular treatment is appropriate or suitable for a particular individual. Ultimately it is the sole responsibility of the medical professional to make his or her own professional judgements, so as appropriately to advise and treat patients. Save for death or personal injury caused by the publisher's negligence and to the fullest extent otherwise permitted by law, neither the publisher nor any person engaged or employed by the publisher shall be responsible or liable for any loss, injury or damage caused to any person or property arising in any way from the use of this book.

A CIP record for this book is available from the British Library.

ISBN-13: 9781439801796

Orders may be sent to: Informa Healthcare, Sheepen Place, Colchester, Essex CO3 3LP, UK
Telephone: +44 (0)20 7017 6682
Email: Books@Informa.com
Website: <http://informahealthcarebooks.com>

Library of Congress Cataloging-in-Publication Data

Pharmaceutical stress testing : predicting drug degradation / edited by Steven W. Baertschi, Karen M. Alsante, Robert A. Reed. -- 2nd ed.

p. ; cm. -- (Drugs and the pharmaceutical sciences series)

Includes bibliographical references and index.

ISBN 978-1-4398-0179-6 (hardback : alk. paper) 1. Drug stability--Testing. I. Baertschi, Steven W. II. Alsante, Karen Mills. III. Reed, Robert A. IV. Series: Drugs and the pharmaceutical sciences (Unnumbered)

[DNLM: 1. Drug Stability. 2. Chemistry, Pharmaceutical--methods. 3. Pharmaceutical Preparations--analysis. QV 754]

RS424.P42 2011

615'.19--dc23

2011017897

For corporate sales please contact: CorporateBooksIHC@informa.com

For foreign rights please contact: RightsIHC@informa.com

For reprint permissions please contact: PermissionsIHC@informa.com

Typeset by Exeter Premedia Services Private Ltd., Chennai, India

Printed and bound in the United Kingdom.

Contents

<i>Contributors</i>	<i>viii</i>
<i>Preface</i>	<i>xi</i>
<i>Acknowledgments</i>	<i>xii</i>
1. Introduction	1
<i>Steven W. Baertschi and Dan W. Reynolds</i>	
2. Stress testing: A predictive tool	10
<i>Steven W. Baertschi, Patrick J. Jansen, and Karen M. Alsante</i>	
3. Stress testing: The chemistry of drug degradation	49
<i>Steven W. Baertschi, Karen M. Alsante, and Dinos Santafricanos</i>	
4. Stress testing: Analytical considerations	142
<i>Patrick J. Jansen, W. Kimmer Smith, and Steven W. Baertschi</i>	
5. Stress testing: Relation to the development timeline	161
<i>Steven W. Baertschi, Bernard A. Olsen, Karen M. Alsante, and Robert A. Reed</i>	
6. Oxidative susceptibility testing	168
<i>Paul Harmon and Giovanni Boccardi</i>	
7. Photostability stress testing	192
<i>Elisa Fasani and Angelo Albini</i>	
8. Practical aspects of conducting photostability stress testing	218
<i>David Clapham, Allen C. Templeton, Lee J. Klein, and Mark H. Kleinman</i>	
9. Role of “mass balance” in pharmaceutical stress testing	233
<i>Mark A. Nussbaum, Andreas Kaerner, Patrick J. Jansen, and Steven W. Baertschi</i>	
10. Solid-state pharmaceutical development: Ensuring stability through salt and polymorph screening	254
<i>Susan M. Reutzel-Edens and Greg A. Stephenson</i>	
11. Solid-state excipient compatibility testing	286
<i>Amy S. Antipas, Margaret S. Landis, and W. Peter Wuelfing</i>	
12. Small molecule parenteral drugs: Practical aspects of stress testing	322
<i>Andreas Abend, Brett Duersch, and Kyle Fiszlar</i>	
13. Stability considerations in development of freeze-dried pharmaceuticals	343
<i>Steven L. Nail</i>	
14. Stress testing of therapeutic monoclonal antibodies	370
<i>Michael R. DeFelippis, Bryan J. Harmon, Lihua Huang, and Muppalla Sukumar</i>	
15. Stress testing of oligonucleotides	391
<i>Daniel C. Capaldi</i>	
16. Stress testing to determine liposome degradation mechanisms	426
<i>Paul R. Meers and Patrick L. Ahl</i>	

17. Stress testing of combination therapies <i>Dan W. Reynolds and Biren K. Joshi</i>	447
18. Rapid stress stability studies for evaluation of manufacturing changes, materials from multiple sources, and stability-indicating methods <i>Bernard A. Olsen, Michael A. Watkins, and Larry A. Larew</i>	460
19. Stress testing as a predictive tool for the assessment of potential genotoxic degradants <i>Steven W. Baertschi, David DeAntonis, Alan P. McKeown, Joel Bercu, Stephen Raillard, and Christopher M. Riley</i>	484
20. The power of computational chemistry to leverage stress testing of pharmaceuticals <i>Donald B. Boyd and Thomas R. Sharp</i>	499
21. Automation in conducting stress testing and excipient compatibility studies <i>Eric Carlson, Patrick J. Jansen, and Christopher Foti</i>	540
22. Use of isothermal microcalorimetry in stress testing <i>Graham Buckton and Simon Gaisford</i>	560
23. Temperature excursions during shipment and storage <i>Manuel Zahn</i>	583
24. Stress testing: Frequently asked questions <i>Steven W. Baertschi, Karen M. Alsante, and Robert A. Reed</i>	594
<i>Index</i>	605

Contributors

Andreas Abend Merck Manufacturing Division, West Point, Pennsylvania, USA

Patrick L. Ahl Vaccine Drug Product Development & New Technologies, Vaccine Bioprocess Research & Development, Merck Research Laboratories, West Point, Pennsylvania, USA

Angelo Albini Dipartimento di Chimica, Università di Pavia, Pavia, Italy

Karen M. Alsante Pfizer, Inc., Groton, Connecticut, USA

Amy S. Antipas Pfizer, Inc., Groton, Connecticut, USA

Steven W. Baertschi Analytical Sciences R&D, Eli Lilly and Company, Indianapolis, Indiana, USA

Joel Bercu Lilly Research Laboratories, Health, Safety and Environmental, Eli Lilly and Company, Indianapolis, Indiana, USA

Giovanni Boccardi Analytical Sciences—LGCR, Sanofi-Aventis, Milan, Italy

Donald B. Boyd Department of Chemistry and Chemical Biology, Indiana University-Purdue University at Indianapolis, Indianapolis, Indiana, USA

Graham Buckton Department of Pharmaceutics, School of Pharmacy, University of London, London, UK

Daniel C. Capaldi Isis Pharmaceuticals, Inc., Carlsbad, California, USA

Eric Carlson Freeslate, Inc., Sunnyvale, California, USA

David Clapham Exploratory Development Sciences, Pharmaceutical Development, Glaxo-SmithKline Pharmaceuticals, Ware, UK

David DeAntonis Pfizer, Inc., Groton, Connecticut, USA

Michael R. DeFelippis Biopharmaceutical Research and Development, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana, USA

Brett Duersch Merck Manufacturing Division, West Point, Pennsylvania, USA

Elisa Fasani Dipartimento di Chimica, Università di Pavia, Pavia, Italy

Kyle Fiszar Merck Manufacturing Division, West Point, Pennsylvania, USA

Christopher Foti Pfizer, Inc., Groton, Connecticut, USA

Simon Gaisford School of Pharmacy, University of London, London, UK

Bryan J. Harmon Biopharmaceutical Research and Development, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana, USA

Paul Harmon Analytical Sciences, Pharmaceutical Sciences and Clinical Supply, Merck & Co. Inc., West Point, Pennsylvania, USA

Lihua Huang Biopharmaceutical Research and Development, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana, USA

Patrick J. Jansen Eli Lilly and Company, Indianapolis, Indiana, USA

Biren K. Joshi Chemical Development, GlaxoSmithKline, Research Triangle Park, North Carolina, USA

Andreas Kaerner Analytical Sciences Research and Development, Lilly Technology Center, Indianapolis, Indiana, USA

Lee J. Klein Pharmaceutical Research & Development, Merck Research Laboratories, Merck & Co., Inc., West Point, Pennsylvania, USA

Mark H. Kleinman GlaxoSmithKline, King of Prussia, Pennsylvania, USA

Margaret S. Landis Pfizer, Inc., Groton, Connecticut, USA

Larry A. Larew Eli Lilly and Company, Indianapolis, Indiana, USA

Alan P. McKeown Pfizer, Inc., Sandwich, UK

Paul R. Meers Department of Chemistry, Medical Technology and Physics, Monmouth University, West Long Branch, New Jersey, USA

Steven L. Nail Baxter Pharmaceutical Solutions, Bloomington, Indiana, USA

Mark A. Nussbaum Chemistry Department, Hillsdale College, Hillsdale, Michigan, USA

Bernard A. Olsen Olsen Pharmaceutical Consulting, LLC, West Lafayette, Indiana, USA

Stephen Raillard XenoPort, Inc., Santa Clara, California, USA

Robert A. Reed Celsion Corporation, Columbia, Maryland, USA

Susan M. Reutzel-Edens Pharmaceutical Sciences R&D, Lilly Research Laboratories, Eli Lilly & Company, Indianapolis, Indiana, USA

Dan W. Reynolds Chemical Development, GlaxoSmithKline, Research Triangle Park, North Carolina, USA

Christopher M. Riley Riley and Rabel Consulting Services, LLC, Maryville, Missouri, USA

Dinos Santaifanos Pfizer, Inc., Groton, Connecticut, USA

Thomas R. Sharp (Emeritus) Pfizer, Inc., Groton, Connecticut, USA

W. Kimmer Smith Analytical Sciences R&D, Eli Lilly and Company, Indianapolis, Indiana, USA

Greg A. Stephenson Eli Lilly & Company, Indianapolis, Indiana, USA

Muppalla Sukumar Biopharmaceutical Research and Development, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana, USA

CONTRIBUTORS

Allen C. Templeton Pharmaceutical Research & Development, Merck Research Laboratories, Merck & Co., Inc., West Point, Pennsylvania, USA

Michael A. Watkins Analytical Sciences R&D, Eli Lilly and Company, Indianapolis, Indiana, USA

W. Peter Wuelfing Basic Pharmaceutical Sciences, Merck Research Laboratories, Merck & Co., Inc., West Point, Pennsylvania, USA

Manuel Zahn 3R Pharma Consulting GmbH, Döbel, Germany

I Preface

Stress testing has long been recognized as an important part of the drug development process. Efforts by the International Conference on Harmonization (ICH) with regard to impurities and photostability have brought an increased regulatory scrutiny of impurities, requiring identification and toxicological qualification at very low levels. Coupled with the fact that the pharmaceutical industry is making major efforts to reduce the time it takes to get products to market, the potential for stability and impurity “surprises” that affect the development timeline has increased dramatically. Stress testing is the main tool that is used to predict stability problems, develop analytical methods, and identify degradation products/pathways. Since there are no detailed regulatory guidelines that direct how stress testing is to be done, nor had there ever been a text/reference book on the subject, stress testing has evolved into an artful science that is highly dependent on the experience of the company or the individuals directing the studies. The first edition of this book on pharmaceutical stress testing focused on providing the readers with a single source reference that benchmarked the best practices of experienced pharmaceutical scientists/researchers. Since the publication of the first edition, the field of pharmaceutical stress testing (also often referred to as “Forced Degradation”) has experienced significant attention, with numerous scientific conferences and scientific publications on the topic. This second edition of the book provides a coherent compilation of this progress and hopefully stimulating the field to continue to rapidly develop.

This book provides readers with a single source reference that benchmarks the current best practices of experienced pharmaceutical scientists/researchers. Questions like “How hot, how long, what conditions are appropriate?” and topics such as mass balance, photostability, oxidative susceptibility, and the chemistry of drug degradation are addressed in an objective, detailed scientific manner, with ample references to relevant guidances and the scientific literature. Stress testing, which encompasses not only the procedures to conduct stress testing but also the science underlying degradation chemistry and chemical stability, is very broad and scientifically complex. Thus, this book seeks to be both a practical and scientific guide that will hopefully stimulate new ideas and further development of the science.

This second edition is directed toward providing both updates of existing topics and extension of coverage into new topics such as oligonucleotides, proteins, physical aspects of stress testing, quality-by-design principles, and expanded coverage of formulated products and diverse dosage forms. Additionally, the connection between stress testing and “real-world” stability is developed through topics related to kinetics, predictive models, and actual conditions experienced by drugs and drug products during shipping and distribution. Technological advances are also discussed with respect to automation approaches to dramatically increase productivity.

This book approaches the topic of stress testing with the underlying theme that stress testing is predictive in nature, that it is multidimensional (analytical, organic, physical, spectroscopic, and pharmaceutical), and that it is an integral part of the drug development process. This undertaking is done with some trepidation, since we realize that discussion of this topic could lead to attempts to formalize or standardize (i.e., regulatory standardization) an area of pharmaceutical science that relies on scientific expertise and flexibility. Each new drug compound, formulation, route of administration, delivery device, etc., has the potential to present unique challenges and unanticipated problems, and should be approached with such a mindset. The pharmaceutical researcher must be free to design and develop new ways to predict and measure stability-related issues through stress testing. Nonetheless, it is hoped that this book will provide a useful and practical scientific guide that is a welcome addition to the library of many pharmaceutical scientists. We are confident that the “artful science” of stress testing will continue to evolve beyond what is represented in this book as other scientists further the science and fill in topics that are poorly covered or missing.

I Acknowledgments

Steven W. Baertschi: I am indebted to my co-workers, especially Patrick J. Jansen and W. Kimmer Smith for helping to develop our strategies for understanding degradation chemistry and for conducting stress testing over the last 18 or so years. I am grateful to Jerry R. Draper, Bradley M. Campbell, and Robert M. Montgomery for all their work on stress testing and degradation studies over the last several years, especially in the development of efficient and effective automation procedures. I want to thank Lindsay N. (Maxwell) Backer and Michael A. Vance for their contributions while they were a part of our group. I also want to thank the numerous scientists at Eli Lilly and Company who have helped me develop this area through their collaborations—the names are too many to mention, but Timothy J. Wozniak and Bernard A. Olsen deserve special acknowledgment and thanks for mentoring me over the years. I am grateful to Eli Lilly and Company, and especially Eugene Inman, for allowing me to focus on an area of pharmaceutical science for an extended period of time and for supporting me in my efforts to assemble two editions of a book devoted to the topic of stress testing. This second edition would not have been possible without the contributions and encouragement of two excellent co-editors, Karen Alsante and Bob Reed, who have been a joy to work with. But most of all I am in debt to my family, Wade, Joey, Jordan, and Tristen, and especially my wife, Cheryl, for graciously putting up with the “stress” resulting from the effort required to complete this book.

Karen M. Alsante: I am grateful for my Pfizer Groton Degradation Group co-workers, especially Todd Hatajik, Dr. Dinos Santafianos, and Todd Zelesky for their hard work and dedication building our predictive degradation approach, experimental protocols, database efforts, and for their support and friendship over the years. I am also extremely appreciative of the management support I have received from Dr. Rich Irwin and Dr. Sarah Kelly of Pfizer Pharmaceutical Sciences Research Science & Technology while working on this project over the past three years. Most importantly, I am very thankful for all the strength and support from my family, Jim, Matthew, and Kristina who put up with all my weekend stress testing review work. In closing, a special thanks to my friend and mentor Steve Baertschi for inviting me as a co-editor on the second edition. It has been such an honor to collaborate with Steve and Bob Reed on this book. We have a unique partnership that dates back to 2000. It has been such a rewarding experience to pioneer this field as a team.

Robert A. Reed: I am indebted to my introduction to degradation chemistry in pharmaceutical products through the many scientific discussions with colleagues at Merck & Company, XenoPort, Inc., and Celsion Corporation. More specifically, scientific discussions with Paul Harmon, Allen Templeton, Pete Wuelfing, Andreas Abend, Qingxi Wang, Brett Duersch, Yun Mao, Anne Payne, Mark Mowery, Lee Klein, Rey Chern, Zack Zhao, Eric Nelson, Randy Seburg, James Qin, John Ballard, Winnie Yin, Sandra Arocho, Bill Bowen—all of Merck; Stephen Raillard, Peadar Cremin, Quincey Wu, Hui Yan, Weihong Gong at XenoPort; and Daishui Su at Celsion, have truly framed my perspective of the chemistries important to pharmaceutical products. It is clear that nothing would have been accomplished without their respective scientific passion for understanding chemical and physical stability issues in pharmaceutical products. I am also greatly indebted to Steve and Karen for the many discussions through the past 10 years on pharmaceutical degradation chemistry, and their commitment to bringing this excellent text to completion. Finally, I am eternally grateful for the support that I have received from my wife Debby, throughout the years and especially during the editing of this book.

1 Introduction

Steven W. Baertschi and Dan W. Reynolds

GENERAL INFORMATION/BACKGROUND

Stress testing has long been recognized as an important part of the drug development process. Efforts by the International Conference on Harmonization (ICH) with regard to impurities (1,2,3,4) and stability (5,6,7) have brought an increased regulatory scrutiny of impurities, requiring identification and toxicological qualification at specified levels. Coupled with efforts by the pharmaceutical industry to reduce the time and cost that it takes to get products to market, the potential for stability and impurity “surprises” that affect the development timeline has increased dramatically. Efforts to improve and streamline processes related to early identification of potential impurity problems are important to the goal of providing new, safe medicines, faster (8).

Stress testing is the main tool that is used to predict stability problems, develop analytical methods, and identify degradation products and pathways. Since there are no detailed regulatory guidelines that describe how to carry out stress testing studies (nor has there been a textbook or reference book devoted to the subject prior to the first edition of this book), stress testing has evolved into an “artful science” that is highly dependent on the experience of the company and of the individuals directing the studies. Questions such as “How hot?”, “How long?”, “What level of humidity?”, “What pH values should be used?”, “What reagents/conditions for oxidative studies should be used?” are faced by every pharmaceutical investigator attempting to carry out stress-testing studies. As will be described in more detail in the section “Historical Context”, this has led to a tremendous variation in stress testing approaches and conditions. An article in 2003 about stress testing (or forced degradation) was even entitled “The Gray Area,” in reference to the vagueness of the current guidelines (9).

The primary focus of this book is to provide a practical and scientific guide for the pharmaceutical scientist to help in designing, executing, and interpreting stress-testing studies for traditional small molecule (typically synthetically prepared) drug substances. Nonetheless, some of the principles and strategies may be generally applicable to large molecules. It is worth noting that the book has been expanded from the first edition to include chapters specifically devoted to large molecules such as proteins/antibodies (chaps. 13 and 14 and 15). Also, while the primary emphasis is on the *chemical* aspects of stress testing, this edition of the book includes significantly expanded coverage of *physical* aspects of stability (see for example, chaps. 10, 13, 16, and 22). On the formulated product side, the focus of this book is on traditional solid oral dosage forms, although some consideration is given to other dosage forms (see chaps. 12, 13, and 16). Detailed consideration of other alternate drug delivery systems (e.g., metered-dose inhalers, transdermal patches, dermal creams, etc.) is beyond the scope of the current edition of this book. Finally, a chapter devoted to the emerging topic of degradation-derived genotoxic impurities and considerations for risk assessment of new drugs and their potential degradation pathways is included in this edition (chap.19).

DEFINITIONS/TERMS

It is important to have a clear definition of terms to facilitate the discussion. In the context of pharmaceuticals, “stress testing” is historically a somewhat vague and undefined term, often used interchangeably with the terms “accelerated stability” and “forced degradation”. A 1980 article by Pope (10) defined accelerated stability testing as “the *validated* method or methods by which product stability may be predicted by storage of the product under conditions, which accelerate change in a defined and predictable manner.” The term “validated” was intended to emphasize that the change occurring under the accelerated conditions must be demonstrated to correlate with normal long-term storage. The United States FDA definition of “accelerated testing” (11) in the February 1987 guideline states that “the term ‘accelerated testing’ is often

used synonymously with ‘stress testing’.” This usage is understandable in which the term “stress testing” is used in many industries to describe testing intended to measure how a system functions when subjected to “an applied force or system of forces” (12). More recently, the ICH introduced an important distinction between the two terms in the context of pharmaceutical stability. The ICH defined “accelerated testing” (13) as:

Studies designed to increase the rate of chemical degradation or physical change of an active drug substance or drug product using exaggerated storage conditions as part of the formal, definitive, storage program. These data, in addition to long-term stability studies, may also be used to assess longer-term chemical effects at nonaccelerated conditions and to evaluate the impact of short-term excursions outside the label storage conditions such as might occur during shipping. Results from accelerated testing studies are not always predictive of physical changes.

An important aspect of this definition is that the studies are part of the “formal, definitive, storage program.” In contrast, ICH, in “Annex 1, Glossary, and Information” of the revised stability guideline (6) defined stress testing (drug substance) as:

Studies undertaken to elucidate the intrinsic stability of the drug substance. Such testing is part of the development strategy and is normally carried out under more severe conditions than those used for accelerated testing.

A more detailed description of stress testing is provided near the beginning of the ICH Stability guideline, under the “Drug Substance” heading:

Stress testing of the drug substance can help identify the likely degradation products, which can in turn help establish the degradation pathways and the intrinsic stability of the molecule and validate the stability indicating power of the analytical procedures used. The nature of the stress testing will depend on the individual drug substance and the type of drug product involved.

Stress testing is likely to be carried out on a single batch of the drug substance. It should include the effect of temperatures (in 10°C increments (e.g., 50°C, 60°C, etc.) above that for accelerated testing), humidity (e.g., 75% RH or greater) where appropriate, oxidation, and photolysis on the drug substance. The testing should also evaluate the susceptibility of the drug substance to hydrolysis across a wide range of pH values when in solution or suspension. Photostability testing should be an integral part of stress testing. The standard conditions for photostability testing are described in ICH Q1B.

Examining degradation products under stress conditions is useful in establishing degradation pathways and developing and validating suitable analytical procedures. However, it may not be necessary to examine specifically for certain degradation products if it has been demonstrated that they are not formed under accelerated or long-term storage conditions.

Results from these studies will form an integral part of the information provided to regulatory authorities.

The description of stress testing was slightly modified in the revised stability guideline from the original description in ICH Q1A (5). The original Q1A description contained this additional paragraph:

Stress testing is conducted to provide data on forced decomposition products and decomposition mechanisms for the drug substance. The severe conditions that may be

encountered during distribution can be covered by stress testing of definitive batches of drug substance.

The ICH definition of stress testing for the drug *product* is as follows (7):

Studies undertaken to assess the effect of severe conditions on the drug product. Such studies include photostability testing (see ICH Q1B) and specific testing on certain products, (e.g., metered dose inhalers, creams, emulsions, refrigerated aqueous liquid products).

From the ICH definition it is clear that there is now a (regulatory) differentiation between “accelerated testing” and “stress testing.” Stress testing is distinguished by both the severity of the conditions and the focus or intent of the results. Stress testing, which is also often referred to as “forced degradation,” is an investigation of the “intrinsic stability” characteristics of the molecule, providing the foundation for developing and validating analytical methods and for developing stable formulations. Stress testing studies are intended to *discover* stability issues, and are therefore *predictive* in nature. Stress testing studies are not a part of the “validated” formal stability program. Rather, pharmaceutical stress testing is a research investigation requiring scientific expertise and judgment. These concepts have ramifications for the design and execution of stress testing studies, which will be explored in more detail later.

HISTORICAL CONTEXT

As discussed above, the terms stress testing and accelerated (stability) testing were often used interchangeably in the pharmaceutical industry. Usually these topics were discussed as part of an overall discussion of drug stability and/or prediction of shelf life (14) although in some cases the focus was on degradation pathways or chemical reactivity/ stabilization (15). In a classic article by Kennon (16), the effect of increasing temperature (from room temperature to 85°C) on the rates of degradation of pharmaceutical products was discussed in the context of predicting shelf life of pharmaceuticals. This article provided the basis for many articles that followed. For example, the articles by Yang and Roy (17) and Witthaus (18) were extensions of Kennon’s original work. Their work led Joel Davis of the FDA to propose what is known as the “Joel Davis rule,” that is, 3 months at 40°C/75% relative humidity is roughly equivalent to 24 months at room temperature (25°C) (19). Interestingly, Carstensen has pointed out that prior to the “Joel Davis rule,” the historical “rule-of-thumb” had been that 5 weeks of storage at 42°C is equivalent to 2 years of storage at room temperature (20). This rule had been derived from work done in 1948 on the stability of vitamin A and it assumes the same activation energy as found for vitamin A. Other important contributions have been made over the years with regard to kinetic evaluations of drug stability from an “accelerated stability” viewpoint (e.g., modification of the Arrhenius equation to include the influence of relative humidity) (21,22,23), but a comprehensive review of the literature related to the kinetics of degradation is not the point of focus here.

It is interesting to consider some of the conditions that have historically been employed in the stress testing of pharmaceuticals, documented both in the “Analytical Profiles of Drug Substances” (24) and by Singh (25). Acidic stress conditions can be found to vary from 0.1 N HCl at 40°C for 1 week (with “negligible degradation”) (26), to 0.1 N HCl at 65°C for 21 days (71.6% degradation) (25), to 0.1 N HCl at 105°C for 2 months (with “considerable degradation”), to 4 N HCl under refluxing conditions for 2 days (66% degradation) (27), to 6.5 N HCl at 108°C for 24 hour (50% degradation), and to concentrated HCl at room temperature (56.5% degradation) (28). Similar elevated temperatures, times, and base strength have been employed for basic stress conditions. For example, conditions can be found to vary from 0.1 N NaOH at 40°C for 1 week (with negligible degradation) (26), to 0.1 N NaOH at 65°C for 21 days (with 100% degradation) (25), to 0.1 N NaOH under refluxing conditions for 2 days (68% degradation) (27),

to 1 N NaOH under boiling conditions for 3 days (7.2% degradation) (29), and to 5 N NaOH under refluxing conditions for 4 hour (100% degradation) (30). In terms of oxidative degradation studies, hydrogen peroxide has been employed at strengths from 0.3% to 30% (31). Studies were often conducted at elevated temperatures, e.g., 37°C for 6 hour [3% hydrogen peroxide, 60% degradation (32)], 50°C for 72 hour (3% hydrogen peroxide, 6.6% degradation), and even refluxing conditions for 30 minute (3% hydrogen peroxide, extensive degradation) (30) or 6 hour (10% hydrogen peroxide, no significant degradation) (33).

As these examples illustrate, historically there has been tremendous variation in the conditions employed in acid/base and oxidative stress testing studies. There has also been tremendous variation in defining the appropriate "endpoint" of the stress testing studies, that is, what length of time (and temperature) or amount of degradation is sufficient to end the stress exposure.

Perhaps the most dramatic variability in stress testing conditions is observed in the photostressing of drugs (34) where the lamps and exposures range from short wavelength Hg arc lamps (254 nm, UVC range) to fluorescent light to "artificial light" to halogen lamps to xenon lamps. The variability of photoexposure during pharmaceutical photostability studies has also been documented by surveys of the pharmaceutical industry (35,36,37).

From the information provided above it is apparent that stress-testing conditions have varied greatly from compound to compound and from investigator to investigator. Extremely harsh conditions have been commonly used in the past to *ensure degradation*, even if the conditions far exceeded plausible exposures.

More recently, several articles relevant to stress testing have appeared in the pharmaceutical literature. A paper by Singh and Bakshi (25) in 2000 provides the most thorough collection of references to various degradation studies of drug products, documenting the diversity of conditions and approaches to stress testing. This paper attempts to provide a classification system (extremely labile, very labile, labile, stable) based on a defined systematic approach. It is not clear from the article on what basis (scientific or otherwise) the classification system was devised; however, the paper does define "endpoints" to stressing (albeit, fairly harsh endpoints), allowing for the conclusion that a particular compound may be regarded as "stable" under a certain set of conditions.

In 1992 (and again in 1994), Boccardi provided some needed guidance on oxidative stress testing by asserting that most pharmaceutical oxidative degradation was the result of autoxidation and that hydrogen peroxide was not a very good reagent to mimic autoxidation processes (38,39). Boccardi was the first to describe the use of radical initiators such as azobisisobutyronitrile (AIBN) for oxidative pharmaceutical stress testing, and he provided a simple procedure with mild conditions he termed "the AIBN test." In 1996, Baertschi presented and discussed an approach to stress testing that had defined limits of harshness and exposure time (40). In 1998, Weiser (41), in discussing the role of stress testing in analytical method development, suggested a set of conditions for performing stress testing that was arguably milder than many of the historical studies cited above. In 2001, Alsante et al. (42) provided a guide to stress testing studies that suggested defined limits to the stress conditions. For example, for acidic and basic stressing, Alsante suggested conditions of 1N HCl and 1N NaOH for a maximum of 1 week at room temperature. In 2002, the views of the Pharmaceutical Research and Manufacturer's Association (PhRMA) were summarized in an article on forced degradation studies (43). The PhRMA article did not discuss specifics of conditions of stress, but rather focused more on what kinds of stress testing should be performed for drug substances and products and on the regulatory requirements.

A survey on the publications on the topic of stress testing/forced degradation studies in more recent years (i.e., 2001–2003) revealed that there was still a tremendous variability in the conditions employed. A few examples will be discussed here, although this discussion is not intended to be an exhaustive review of the literature.

A degradation study of haloperidol utilized 1 M HCl and 1 M NaOH (refluxed for 5 hour), and 30% hydrogen peroxide (70°C for 5 hour) for the most stressful conditions of the study (44).

These conditions appear to have been chosen to enable production of known degradation products (six degradation products shown) to facilitate HPLC method validation efforts. A degradation study of ibuprofen produced 13 degradation products, several of which had never before been detected (45). In this study, oxidative studies were carried out utilizing potassium permanganate (0.05 M) at room temperature up to 16 hour in 0.5 M NaOH; up to 33% hydrogen peroxide at room temperature for 22 hour; and potassium dichromate (0.1 N) at room temperature up to 14 days in 0.5 M HCl. Solid-state studies utilized 50°C up to 8 months and 100°C up to 16 hour to detect volatile degradation products. An NMR study of the aqueous degradation of isophosphoramidate mustard was conducted in buffered aqueous solutions in the pH range of 1–13 (46). The degradation of sumatriptan in 0.1 N HCl, 0.1 N NaOH, and in 3% hydrogen peroxide was studied using LC/MS and LC/MS/MS (47). The solutions were heated at 90°C for 30 minute to 9 hour. Photostability was assessed by exposure to UV irradiation at 254 nm for 24 hour (no indication of irradiation intensity). A study of the major oxidative degradation products of SCH56592 was conducted by exposure of the drug substance in the solid state to 150°C for 12 days with identification of the major products using LC-MS and LC-NMR (48). Singh et al. describe stress degradation studies of ornidazole (49) and prazosin, terazosin, and doxazosin (50) under conditions designed to be in “alignment” with the ICH Stability guideline (Q1A2). In the case of ornidazole, significant degradation was seen under acidic conditions of 0.1 M HCl to 5 M HCl at 80°C for 12 to 72 hour, although no degradation products were detected (presumably because of degradation to non-chromophoric products). Studies under basic conditions of 0.1 M NaOH at both 80°C and 40°C revealed complete degradation at time zero. Milder studies were then conducted at pH 8 and 40°C. Oxidative studies involved 3% and 30% hydrogen peroxide at room temperature for 8 hour, with losses of 8% and 53% of the parent, respectively. Photodegradation studies utilized Option 2 of the ICH Q1B photostability guideline (7) with exposures up to 30 days at 7000 lux (over 5 million lux hour exposure). Similar conditions were employed for prazosin, terazosin, and doxazosin.

In the examples of stress testing studies cited above, it is apparent that there has been a great diversity of conditions employed to induce degradation, although the diversity is arguably less than was observed prior to publication of the ICH guidances. This continued diversity of approach could be interpreted in a couple of ways. One interpretation is that stress-testing studies are inherently a research undertaking, and therefore flexibility and scientific judgment are required, leading to diverse conditions and approaches. Another interpretation is that there is (appropriately or inappropriately) very little guidance (either regulatory or in the scientific literature) on the specifics of the conditions or appropriate endpoints of pharmaceutical stress testing. We assert that both interpretations are valid. The goal of the first edition of this book (published in 2005) was to provide, in one source, in-depth scientific guidance to the researcher to enable sound, practical, and reasonably consistent approaches to pharmaceutical stress testing. It is hoped that this second edition will provide updated and expanded guidance for stress testing and the associated science that will prove useful to the industry and to the field of pharmaceutical stability. It is worth noting here that while stability/degradation-related concerns are not new, the regulatory landscape is still evolving, particularly with respect to the potential for the formation of degradation-derived genotoxic impurities. Chapter 19 deals directly with this emerging topic, providing a useful reference for the reader.

REGULATORY CONTEXT

The available regulatory guidances do not explicitly require stress testing be performed or reported at the Phase 1–2 IND stages, although stress testing is encouraged to facilitated selection of stability-indicating methods (51). Experience has shown, however, that regulatory authorities may still ask questions concerning results from stress testing as early as a Phase 1 IND, especially where potentially toxic (e.g., genotoxic) degradation products are possible. The guidance does require drug substance stress testing for the Phase 3 IND and suggests these studies be

conducted on drug products. At Phase 3, the guidance strongly suggests, but does not always require, that degradation products detected above the ICH identification thresholds during formal stability trials should be identified. For an NDA, the guidance requires a summary of drug substance and drug product stress studies including elucidation of degradation pathways, demonstration of the stability-indicating nature of analytical methods, and identification of significant degradation products (52,53). Stressing the drug substance under hydrolytic, oxidative, photolytic, and thermolytic conditions in solution and the solid state is required. The design of drug product studies is formulation dependent and is left to the discretion of the applicant.

Although not necessarily directly related to stress testing, the guidance also requires demonstration and/or a summary of an investigation of mass balance (6) in degraded samples from formal stability trials, an assessment of the drug's stereochemical stability (54), and distinguishing drug related and nondrug related degradation products (4). However, these issues can often be addressed in stress studies fulfilling both scientific need and regulatory requirements. The predictive nature of well-conducted stress studies can forewarn of potential problems in these areas early-on, facilitating appropriate and efficient changes in the development strategy, if required.

The guidance suggests the analytical assumptions made when determining mass balance should be explained in the registration application (4). Failure to demonstrate mass balance may be acceptable provided a thorough investigation has been conducted to understand the chemistry of the molecule (5). Examining mass balance in stressed samples can reveal the need for better analytical methodology from the start (55).

The guidance recommends treating chiral impurities as though they were achiral impurities with the caveat that the ICH identification and qualification thresholds may not apply for analytical reasons (54). Experimental demonstration that stereoisomers of the drug substance and its degradation products do not form during stress studies, especially when combined with mechanistic understanding, can eliminate the need for analytical monitoring of these potential impurities during formal stability trials. Experience has shown that merely arguing a particular chiral center is unlikely to invert on strictly theoretical grounds may not be acceptable to the (U.S.A.) FDA and many other regulatory agencies worldwide.

Differentiation between drug-related and nondrug-related degradation products can be achieved with stress studies of the drug substance, drug product (including excipient compatibility studies), and placebo (i.e., the formulation minus the active). These studies should allow discrimination between synthetic process impurities, excipients, degradation products derived from excipients alone, and drug-related degradation products including drug–excipient combinations.

The guidance suggests that the potential for reactions between active ingredients in combination products should be investigated (52,56). For a triple combination tablet formulation, the FDA suggested stressing the three actives together under conditions usually applied to a single drug substance. These studies were conducted and reported in the NDA. Chapter 17 addresses combination products in more detail and provides general suggestions for the content of the NDA drug substance and product regulatory modules.

The available guidance specifies identification thresholds for degradation products observed in formal stability samples of the drug substance and product that depend upon the dosage (1,2,3,4). Consideration for not identifying degradation products that are detected at the threshold levels is given for degradation products which are unstable (4). In those cases, a summary of the efforts to isolate and identify the unstable degradation product may suffice.

CONCLUSION

Stress testing is the foundational stability investigation, facilitating the development of valid stability-indicating analytical methods, and enabling both prediction of stability problems and meaningful long-term stability assessments. As stated earlier in this chapter, the goal of this book is to provide a practical and scientific guide for the pharmaceutical scientist to help in designing, executing, and interpreting stress testing studies. This second edition of the book is

expanded to include additional areas of coverage of proteins, oligonucleotides, some physical aspects of stability, considerations for potential genotoxic risk assessment, and some dosage forms other than solid oral dosage forms. In addition, every chapter has been significantly updated and revised. It is hoped that this expanded and updated coverage will provide a more comprehensive resource for pharmaceutical researchers worldwide.

REFERENCES

1. International Conference on Harmonisation, Impurities in New Drug Substances, Q3A, January 1996.
2. International Conference on Harmonisation, Impurities in New Drug Substances, Q3A(R2), October 2006.
3. International Conference on Harmonisation, Impurities in New Drug Products, Q3B, November 1996.
4. International Conference on Harmonisation, Impurities in New Drug Products, Q3B(R2), June 2006.
5. International Conference on Harmonisation, "Stability Testing of New Drug Substances and Products", Q1A, September 1994.
6. International Conference on Harmonisation, "Stability Testing of New Drug Substances and Products," Q1A(R2), February 2003.
7. International Conference on Harmonisation, "Stability Testing: Photostability Testing of New Drug Substances and Products", Q1B, November 1996.
8. Görög S. New safe medicines faster: the role of analytical chemistry. *Trends Anal Chem* 2003; 22: 7–8.
9. Dubin CH. The Gray Area. *Pharma Formulation Qual* 2003: 22–9.
10. Pope DG. Accelerated stability testing for prediction of drug product stability. *Drug and Cosmet Ind* 1980: (Part 1) 54–62, (Part 2) 48–116.
11. Center for Drugs and Biologics. Guideline for Submitting Documentation for the Stability of Human Drugs and Biologics. Rockville, MD: FDA, Department of Health and Human Services, 1987: 2.
12. The American Heritage, 4th edn. Boston, MA: Houghton Mifflin Company, Copyright © 2000 by Houghton Mifflin Company.
13. Reference ICH-Q1A, September 1994. (For drugs to be stored at room temperature, i.e., 25°C, accelerated testing is defined as 40°C/75% relative humidity. For other storage conditions accelerated testing is to be carried out at 15°C above the long-term storage temperature.)
14. (a) Kulschreshtha HK. Use of kinetic methods in storage stability studies on drugs and pharmaceuticals. *Defence Sci J* 1976; 26: 189–204; (b) Witthaus G. Accelerated storage tests: predictive value. In Breimer DD, Speiser P, eds. *North-Holland Biomedical Press*, 1981: 275–90; (c) Carstensen JT. *Drug Stability. Principles and Practices*, 2nd edn, James TS, ed., New York: Marcel Dekker, 1995.
15. (a) Schou SA. Decomposition of pharmaceutical preparations due to chemical changes. *Am J Hosp Pharm* 1960; 17: 153–61; (b) Stewart PJ, Tucker IG. Prediction of drug stability. Part 1. Mechanism of drug degradation and basic rate laws. *Aust J Hosp Pharm*, 1984; 14: 165–70; (c) Stewart PJ, Tucker IG. Prediction of drug stability. Part 2. Hydrolysis. *Aust J Hosp Pharm* 1985; 15: 1,11–16; (d) Stewart PJ, Tucker IG. Prediction of drug stability. Part 3. Oxidation and photolytic degradation *Aust J Hosp Pharm* 1985; 15: 111–17.
16. Kennon L. *J Pharm Sci* 1964; 53: 815–18.
17. Yang W-H, Roy SB. Projection of tentative expiry date from one-point accelerated stability testing. *Drug Dev Ind Pharm* 1980; 6: 591–604.
18. Witthaus G. Accelerated Storage Tests: Predictive Value. *Topics in Pharmaceutical Sciences*, Breimer DD, Speiser P, eds., New York: Elsevier 1981: 275–90.
19. Davis JS. Criteria for Accelerated Stability Testing, presented at the FDA/ASQC Seminar, March 11, Chicago, IL 1991.
20. Carstensen JT. *Drug Stability. Principles and Practices*, 2nd edn., James TS, ed., New York: Marcel Dekker, 1995: 3–4.
21. Waterman KC, Adami RC. Accelerated aging: prediction of chemical stability of pharmaceuticals, *Int J Pharm*. 2005; 293: 101–25.
22. Waterman KC. Understanding and predicting pharmaceutical product shelf-life. In Huynh-ba K, ed., *Handbook of Stability Testing in Pharmaceutical Development: Regulations, Methodologies, and Best Practices*, Berlin: Springer, Chapter 6, 2008: 115–35.
23. Waterman KC, Colgan ST. A science-based approach to setting expiry dating for solid drug products. *Regulatory Rapporteur* 2008; 5: 9–14.
24. Florey K, Brittain HG, eds. *Analytical Profiles of Drug Substances*, Vols. 1–25, New York: Academic, 1972–1998.

25. Singh S, Bakshi M. Guidance on conduct of stress tests to determine inherent stability of drugs. *Pharma Technol Online* 2000; 1-14.
26. Bridle JH, Brimble MT. A stability indicating method for dipyridamole. *Drug Dev Ind Pharm* 1993; 19: 371-81.
27. Padmanabhan G, Becue I, Smith JB. Cloquinol. In Florey K, ed. *Analytical Profiles of Drug Substances*, vol. 1, New York: Academic, 1989: 76-7.
28. Gröningsson K, Lindgren J-E, Lundbeg E, Sandberg R, Wahlén A. Lidocaine base and hydrochloride. In Florey K, ed. *Analytical Profiles of Drug Substances*, Vol. 14. New York: Academic, 1985: 226-7.
29. Lagu AL, Young R, McGonigle E, Lane PA. High performance liquid chromatographic determination of suprofen in drug substance and capsules. *J Pharm Sci* 1982; 71: 85-8.
30. Muhtadi FJ. Analytical profile of morphine. In Florey K, ed. *Analytical Profiles of Drug Substances*, Vol. 17. New York: Academic, 1988: 309.
31. Maron N, Wright G. Application of photodiode array UV detection in the development of stability-indicating LC methods: Determination of mefenamic acid. *J Pharm Biomed Anal* 1990; 8: 101-5.
32. Nassar MN, Chen T, Reff MJ, Agharkar SN. Didanosine. In Brittain HG, ed. *Analytical Profiles of Drug Substances and Excipients*, Vol. 22. New York: Academic, 1993: 216-19.
33. Johnson BM, Chang P-TL. Sertraline hydrochloride. In Brittain HG, ed. *Analytical Profiles of Drug Substances and Excipients*, vol. 24. New York: Academic, 1996: 484.
34. Singh S, Bakshi M. Guidance on conduct of stress tests to determine inherent stability of drugs. *Pharm Technol Online* 2000; 1-14.
35. Anderson NH, Johnston D, McLelland MA, Munden P. Photostability testing of drug substances and drug products in UK pharmaceutical laboratories. *J Pharm Biomed Anal* 1991; 9: 443.
36. Thoma K. Survey of twenty German manufacturers. In Tønnesen H, ed., *Photostability of Drugs and Drug Formulations*. London: Taylor & Francis, 1996: 136-7.
37. (a) Thatcher SR, Mansfield RK, Miller RB, Davis CW, Baertschi SW. Pharmaceutical photostability: a technical and practical interpretation of the ICH guideline and its application to pharmaceutical stability: Part I. *Pharm Technol* 2001; 25: 98-110; (b) Thatcher SR, Mansfield RK, Miller RB, Davis CW, Baertschi SW. Pharmaceutical photostability: a technical and practical interpretation of the ICH guideline and its application to pharmaceutical stability: Part II. *Pharm Technol* 2001; 25: 50-62.
38. Boccardi G, Deleuze C, Gachon M, Palmisano G, Vergnaud JP. *J Pharm Sci* 1992; 81: 183-5.
39. Boccardi G. *Il Farmaco* 1994; 49: 431-5.
40. Baertschi SW. The Role of Stress Testing in Pharmaceutical Product Development, presented at the American Association of Pharmaceutical Scientists Midwest Regional Meeting, Chicago, IL, May 20 1996.
41. Weiser WE. Developing analytical methods for stability testing. *Pharma Technol* 1998; 22: 20-9.
42. Alsante KM, Friedmann RC, Hatajik TD, et al. Degradation and impurity analysis for pharmaceutical drug candidates. In Ahuja S, Scypinski S, eds., *Handbook of Modern Pharmaceutical Analysis*. New York: Academic, vol. 3, 2001: 85-172.
43. Reynolds DW, Facchine KL, Mullaney JF, et al. Available guidance and best practices for conducting forced degradation. *Stud Pharm Technol* 2002; 26.
44. Trabelsi H, Bouabdallah S, Bouzouita K, Safta F. Determination and degradation study of haloperidol by high performance liquid chromatography. *J Pharm Biomed Anal* 2002; 29: 649-57.
45. Caviglioli G, Valeria P, Brunella P, Sergio C, et al. Identification of degradation products of Ibuprofen arising from oxidative and thermal treatments. *J Pharm Biomed Anal* 2002; 30: 499-509.
46. Breil S, Martino R, Gilard V, Malet-Martino M, Niemeyer U. Identification of new aqueous chemical degradation products of isophosphoramidate mustard. *J Pharm Biomed Anal* 2001; 25: 669-78.
47. Xu X, Bartlett MG, Stewart JT. Determination of degradation products of sumatriptan. *J Pharm Biomed Anal* 2001; 26: 367-77.
48. Feng W, Liu H, Chen G, et al. Structural characterization of the oxidative degradation products of an antifungal agent SCH56592 by LC-NMR and LC-MS. *J Pharm Biomed Anal* 2001; 25: 545-57.
49. Bakshi M, Singh B, Singh A, Singh S. The ICH guidance in practice: stress degradation studies on ornidazole and development of a validated stability-indicating assay *J Pharm Biomed Anal* 2001; 26: 891-97.
50. Ojha T, Bakshi M, Chakraborti AK, Singh S. The ICH guidance in practice: stress decomposition studies on three piperazinyl quinazoline adrenergic receptor-blocking agents and comparison of their degradation behavior. *J Pharm Biomed Anal* 2003; 31: 775-83.
51. FDA: Guidance for Industry: INDs for Phase 2 and 3 Studies; Chemistry, Manufacturing, and Controls Information (Issued May 2003).

52. Submitting Documentation for the Stability of Human Drugs and Biologics (CDER, Issued February (1987)).
53. While the 1987 FDA guidance (see previous reference) may be outdated, the same requirements are found in current ICH guidance, including Q1A/Q1B, Q2A/Q2B, Q3A/Q3B, and M4Q.
54. International Conference on Harmonisation; Guidance on Q6A Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances. December 2000.
55. Baertschi SW. Analytical methodologies for discovering and profiling degradation-related impurities. Trends Anal Chem 2006; 25: 758–67.
56. International Conference on Harmonisation, Pharmaceutical Development, Q8, November 2005.

2 | Stress testing: A predictive tool

Steven W. Baertschi, Patrick J. Jansen, and Karen M. Alsante

INTRODUCTION

As described in chapter 1, stress testing is the main tool that is used to predict stability-related problems, develop analytical methods, and identify degradation products and pathways. Stability-related issues can affect many areas, including the following:

- Analytical methods development
- Formulation and packaging development
- Appropriate storage conditions and shelf-life determination
- Safety/toxicological concerns
- Salt selection/polymorph screening
- Manufacturing/processing parameters
- Absorption, distribution, metabolism, and excretion (ADME) studies
- Environmental assessment

It is worth discussing briefly each of these stability-related areas.

Analytical Methods Development

In order to assess the stability of a compound, one needs an appropriate method. The development of a stability-indicating analytical method, particularly an impurity method, is a “chicken and egg” type of problem. That is, how does one develop an impurity method to detect degradation products when one does not know what the degradation products are? Stress-testing studies can help to address this dilemma. Stressing the parent compound under particular stress conditions can generate samples containing degradation products. These samples can then be used to develop suitable analytical procedures. It is important to note that the degradation products generated in the stressed samples can be classified as “potential” degradation products that may or may not be formed under relevant storage conditions. It is also important to note that not all relevant degradation products may form under the stress conditions. Both accelerated and long-term testing studies of the drug substance and formulated drug product are used to determine which of the potential degradation products actually form under normal storage conditions and are, therefore, relevant degradation products. The strategy for developing a stability-indicating method is described in detail in chapter 4.

Formulation and Packaging Development

The knowledge gained from stress testing is useful for formulation and packaging development. Well-designed stress-testing studies can determine the susceptibility of a compound to hydrolysis, oxidation, photochemical degradation, and thermal degradation. This information is then taken into consideration when developing the formulation and determining the appropriate packaging. For example, if stress-testing studies indicate that a compound is rapidly degraded in acid, then consideration might be given to developing an enteric-coated formulation that protects the compound from rapid degradation in the stomach. If a compound is sensitive to hydrolysis, then packaging that protects from water vapor transmission from the outside may be helpful to ensure long-term storage stability. Alternatively, if the compound is sensitive to base-catalyzed degradation, then a formulation with a slightly acidic microenvironment might be needed. Other degradation mechanisms [e.g., oxidative degradation (see chap. 6)] or photodegradation (see chap. 7) can also be prevented or minimized by the use of appropriate packaging and/or formulation. Knowledge of potential drug–excipient interactions

is also critical to developing the best formulation, and therefore it is also important to conduct drug-excipient compatibility studies and formulated product stress-testing studies (see chap. 11).

Appropriate Storage Conditions and Shelf-Life Determination

Determining appropriate storage conditions for a drug substance or product requires knowledge of conditions that induce degradation and the degradation mechanisms. Most of this information can be obtained from stress-testing studies combined with accelerated stability testing. Accurate shelf-life predictions, however, are best made with data from formal long-term stability studies, although recent studies utilizing an “accelerated stability assessment protocol” have demonstrated a high degree of kinetic predictability (1,2,3).

Safety/Toxicological Concerns

Stress-testing studies are useful for assessing whether known toxic compounds or potential genotoxic compounds are formed by degradation of the parent drug (see chap. 19 for a discussion of the relationship between stress testing and the potential formation of genotoxic degradation products). If the formation of (a) toxic compound(s) is possible, steps can be taken early on to inhibit the formation of the toxic compound(s) and to develop sensitive analytical methods to accurately detect and quantify the formation. Stress-testing studies can also facilitate preparation/isolation of a degradation product for toxicological evaluation when synthetic preparation is not feasible.

Salt Selection/Polymorph Screening

Stress-testing studies can help the salt and polymorph selection process by providing rapid information related to chemical and physical stability. As discussed in more detail in chapter 10, the chemical and physical stability of different salt and polymorphic forms can be dramatically different, highlighting the importance of using stability as part of the rationale for selection. The importance of such considerations is illustrated by the estimation that 50% of all drug molecules are administered as salts (4), and this percentage may be growing due to the increasing need to improve solubility by salt formation.

Manufacturing/Processing Parameters

Degradation can also occur during manufacturing or processing steps. Knowledge of conditions that lead to degradation of the parent compound can help in designing appropriate controls/conditions during manufacturing/processing. For example, if a compound is susceptible to degradation at low pH, then either the manufacturing steps under low pH conditions can be avoided or the time and/or temperature can be more carefully controlled to minimize the degradation. It is not uncommon to observe degradation during formulation processing, for example, wet granulation, milling, etc. An understanding of the degradation that may occur during the formulation processing steps can help in choosing conditions to ensure maximum stability of the drug substance (e.g., oxidative susceptibility may lead to the use of processing in an inert-gas atmosphere; hydrolytic instability may lead to the elimination of wet granulation processes in favor of drug processing conditions such as direct compression or roller compaction).

ADME Studies

ADME characteristics of a drug are extensively studied prior to marketing. These studies typically involve identification of the major metabolites, a process that can be difficult owing to the complex matrix (living organism) and often very low levels. Occasionally, degradation products detected in stress-testing studies are also metabolites. In these cases, it is usually easier to generate larger quantities of the metabolite for characterization using the stress

condition rather than isolate it from the living organism. It is also possible that nonenzymatic degradation can occur *in vivo*, and therefore an understanding of what degradation pathways might be relevant under physiological conditions can be important to understanding the ADME of a new drug.

Environmental Assessment

The environmental assessment deals with the fate of the drug in the environment. The information gained from stress testing can be useful for designing and interpreting environmental studies, as the degradation of the drug in the environment will often be similar to degradation observed during stress-testing studies (e.g., hydrolytic, photolytic, and oxidative degradation). Knowledge of the degradation chemistry of pharmaceuticals is also useful in designing effective wastewater treatments for destroying the drug compound in cost effective and environmentally friendly ways (5,6).

PREDICTIVE VS. DEFINITIVE

It is important to remember that stress testing is *predictive* in nature (as opposed to *definitive*). Stress testing is a research tool that is used to discover *potential* stability issues with a drug molecule, providing the scientific foundation for developing stability-indicating methods. Accelerated and long-term stability testing can then utilize the stability-indicating methods in a more formal and definitive manner to generate specific and quantitative information related to the formation of degradation products, rates of degradation, effects of packaging, and ultimately shelf-life. Thus, the degradation products formed under stress conditions may or may not be relevant to the actual storage conditions of the drug substance and/or to the degradation chemistry of the drug product. This reality is reflected in the International Conference on Harmonization (ICH) definition of stress testing (7), where it is stated:

Examining degradation products under stress conditions is useful in establishing degradation pathways and developing and validating suitable analytical methods. However, such examination may not be necessary for certain degradation products if it has been demonstrated that they are not formed under accelerated or long term storage conditions.

Therefore, the degradation products formed during stress testing can be thought of as potential degradation products. Ideally, stress conditions should result in the formation of all potential degradation products that could occur during long-term storage and distribution. The “actual” degradation products that occur during long-term storage or shipping (as revealed by accelerated testing and long-term stability studies) should thus be a subset of the potential degradation products. This concept is illustrated in Figure 1. The overall strategy of stress testing is, therefore, to predict potential issues related to stability of the molecule—either as the drug substance alone or as a formulated product. The potential issues discovered during stress testing then form the basis for development of the overall control strategy to ensure stability throughout the shelf-life of the drug substance and product. This strategy is outlined in Figure 2, and the principles underlying this strategy are analogous to those delineated by the “quality-by-design” (QbD) construct (8–11).

As shown in Figure 1, the overall strategy is similar for both the drug substance and the product. The strategy begins with stress testing of the drug substance followed by analysis using discriminating or “screening” methods (12,13). Such methods should be capable of separating and detecting a broad range of degradation products and can be used for degradation and impurity investigations. In practice, RP-HPLC with UV detection is by far the most common analytical technique currently used for the detection of impurities (13–16). A discriminating RP-HPLC method utilizing a broad gradient elution is recommended for covering a wide polarity range. Other separation techniques or detection modes may be employed, but the key concept is to develop and use a methodology that will maximize separation and provide the

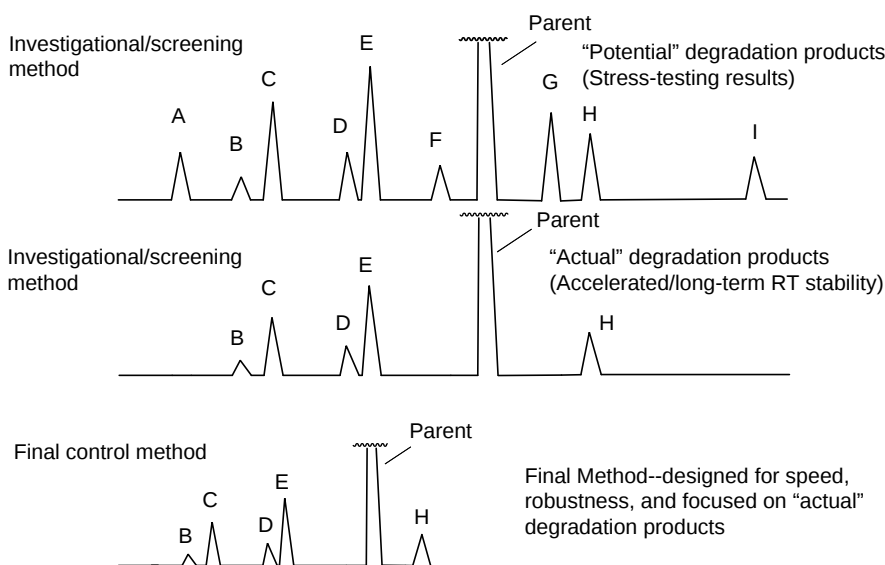


Figure 1 Schematic illustration of hypothetical chromatograms from stress testing (*upper*) and accelerated or long-term stability studies. Peaks A–I represent all the degradation products from stress-testing studies under various stress conditions and are therefore classified as potential degradation products. Peaks B–E and H represent the products that form at significant levels during formal stability studies and are therefore classified as the actual degradation products.

most universal detection of the parent and degradation products. The screening method can be developed/optimized by analysis of partially degraded samples and the use of standard method development procedures and tools (17a,b). The analysis of stressed samples should reveal the potential degradation products formed under the various stress conditions, and the focus should be on the “major” degradation products formed (refer to the section “Intrinsic Stability: Structures of the Major Degradation Products” or Alsante et al. (18) for definition of major degradants). Accelerated testing and analytical evaluation using the same broad screening method can determine the actual degradation products. Methods designed to separate and detect only the significant degradation products (i.e., those that form at significant levels under accelerated and long-term storage conditions) can then be developed and optimized. Such methods, which have been referred to elsewhere as “focused” methods (12,13), are designed for regulatory registration in the marketing application and use in quality control laboratories for product release and stability. For additional discussion of the analytical aspects of stress testing, see chapters 4 and 9.

The information gathered during stress testing of the drug substance should be used to guide the formulation of the drug product. As described in detail in chapter 11, drug-excipient compatibility studies (19–22) can be performed to determine whether or not individual excipients, excipient blends, or trial formulations have any significant adverse interactions with the parent drug. A broad screening method such as that developed for drug substance stress testing should be used for the analytical evaluation of such studies. As discussed in chapter 22, microcalorimetric techniques may also be useful for the analysis of drug–excipient interactions (23–26). Once a suitable formulation has been developed, stress-testing studies can be performed on the formulation and any resulting degradation products can be compared with the degradation products formed during stress-testing studies of the drug substance alone. In an analogous manner to the strategy for the drug substance, the actual degradation products can be determined via accelerated and long-term stability studies, and focused methods can be developed for regulatory registration and use in quality control laboratories for product release and stability (Fig. 2).

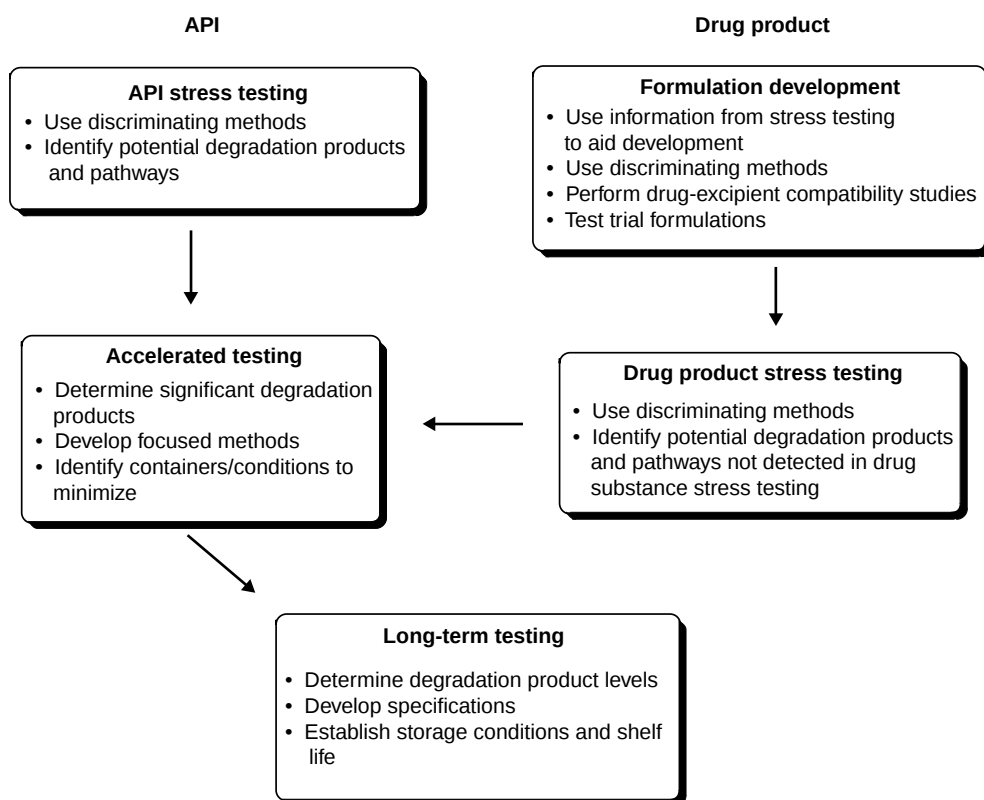


Figure 2 Overall strategy for the prediction, identification, and control of stability-related issues.

The key to the strategy outlined in Figure 2 is to have well-designed stress-testing studies that form all potential degradation products. ICH defines stress testing as an investigation of the “intrinsic stability” characteristics of the molecule. As the term intrinsic stability appears to be foundational to the understanding of stress testing, yet has no clear definition, it is worth discussing further here. The concept of intrinsic stability has four main aspects:

1. Conditions leading to degradation
2. Rates of degradation (relative or otherwise)
3. Structures of the major degradation products
4. Pathways of degradation (including understanding the atoms or functional groups in the chemical structure of the drug molecule that are susceptible to degradation)

Stability-related issues can be identified or predicted once these four areas have been investigated and understood. It is worth considering in more detail the four main aspects of intrinsic stability mentioned above.

INTRINSIC STABILITY: CONDITIONS LEADING TO DEGRADATION

QbD Considerations: Building a Sound Degradation Knowledge Space

As described in the PhRMA “Available guidance and best practices” article on forced degradation studies (27), stress testing should include conditions that examine specifically for four main pharmaceutically relevant degradation mechanisms (12): (i) thermolytic, (ii) hydrolytic, (iii) oxidative, and (iv) photolytic. The potential for these degradation pathways should be assessed both in the

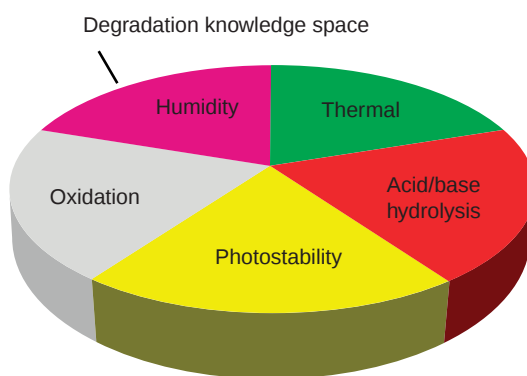


Figure 3 Conditions recommended for stress-testing studies to develop a complete QbD knowledge space.

drug substance and the formulated product (and/or drug– excipient mixtures). These mechanisms can be assessed in a systematic way (providing the basis for understanding intrinsic stability) by exposure to stress conditions of heat, humidity, photostress (UV and VIS), oxidative conditions, and aqueous conditions across a broad pH range (see Fig. 3 for a representation of these concepts).

QbD is defined by ICH as a “systematic approach to pharmaceutical development that begins with predefined objectives and emphasizes product and process understanding based on sound science and quality risk management” (8,28). The concepts of QbD (i.e., developing a thorough knowledge to allow the definition of an acceptable “design space” within which assurance of quality is maintained) can be utilized to provide a useful construct for understanding how stress testing can provide the scientific foundation for analytical method development and the development of control strategies for stability.

The base knowledge acquired through stress-testing results can be thought of as a “knowledge space,” which can be related back to the concept of intrinsic stability used by ICH in the definition of stress testing (see above). The integrity of the degradation knowledge space is dependent on the scientific validity and quality of the investigation of all likely modes of degradation from stress testing under relevant conditions. In summary, stress testing should identify all the reasonably possible degradation products that can result from real-world conditions; such products can be thought of as potential degradation products. The actual degradation products that form for a particular drug will be dependent on variables such as physical state, the dosage form, packaging, and storage conditions, and should be a subset of the potential degradation products. In QbD terminology, the design space can be thought of as the combination of these variables that provide for acceptable stability on accelerated/long-term room temperature stability studies, limiting the formation of actual degradation products to safe and acceptable levels over the shelf-life. While not strictly a QbD concept, a control space can also be envisioned, which defines the optimum set of these variables for the optimum stability in final packaging/storage conditions. A representation of this degradation QbD paradigm is shown in Figure 4, building on the cartoon representation shown in Figure 1.

The integrity of the design and control spaces is dependent upon the integrity and thoroughness of the degradation knowledge space from stress testing. If there are gaps or missing pieces of information in the knowledge space, these gaps can be conceptualized as “holes” in the cartoon representation of the knowledge space (illustrated in Fig. 5). It is important to understand what some of these holes are so that they can be avoided.

Incomplete, poorly designed, or poorly performed stress-testing studies can result in the lack of detection of a significant degradation pathway, and therefore create an incomplete understanding of the degradation chemistry of the drug. Full coverage of hydrolytic, oxidative, thermolytic, and photolytic stress conditions is critical to making sure the degradation

knowledge space is complete. An important part of this “coverage” is the analytical methodology used to separate and detect degradation of the parent drug and the resulting products (12–30). Poor analytical methodology can result in lack of separation of degradation products from the parent, from each other, or lack of detection altogether (e.g., from poor choice of detector, nonelution, volatility, or poor chromatography) (31).

It is also critical to remember that interaction of the drug substance with the formulation and packaging materials can result in degradation pathways not present in the drug substance alone. This can result from a variety of sources including drug-excipient reactions, impurities from excipients or packaging (32), changes in protonation state of the drug substance induced by the formulation (33), formation of the less stable amorphous form during the formulation process, or deliquescence lowering caused by the formulation (34,35).

Thermolytic Degradation

Thermolytic degradation is usually thought of as degradation caused by exposure to temperatures high enough to induce covalent bond breakage, that is, pyrolysis. For the purposes of simplification (although, admittedly, perhaps oversimplification) in the context of drug degradation, we will use the term thermolytic to describe reactions that are driven by heat or temperature, especially in the solid state. Thus, any degradation mechanism that is enhanced at elevated temperatures can be considered a “thermolytic pathway.” The following list of degradation pathways, while not an exhaustive list, can be thought of as thermolytic pathways: hydrolysis/dehydration, isomerization/epimerization, decarboxylation, rearrangements, and some kinds of polymerization reactions. Note that hydrolytic reactions are actually a *subset* of thermolytic pathways using this construct. In addition, note that oxidative and photolytic

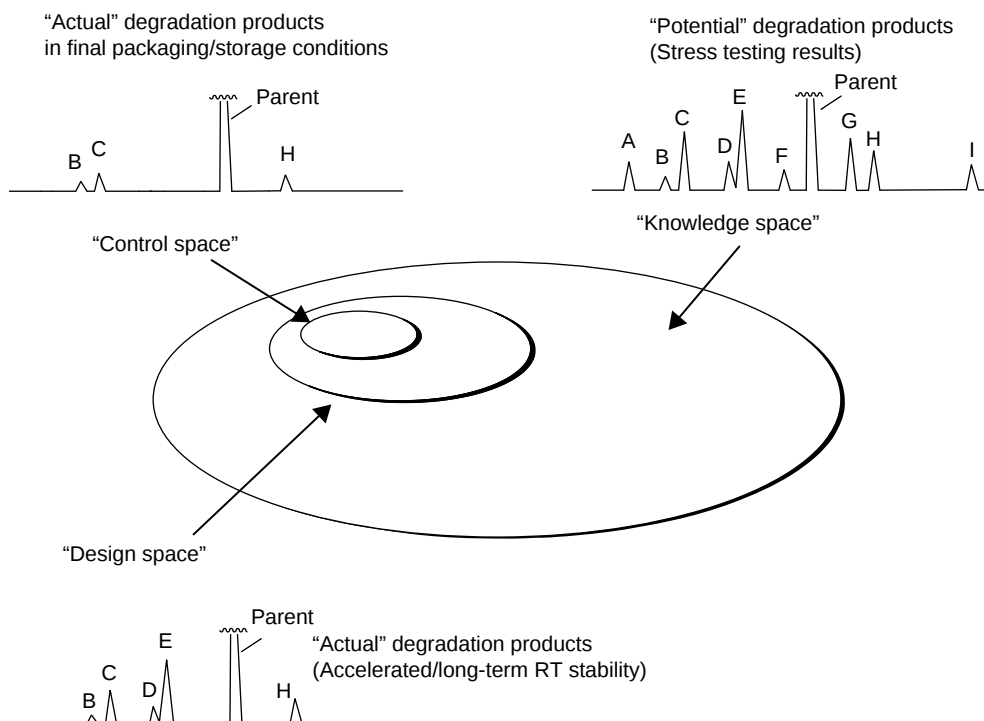


Figure 4 Conceptual illustration of the application of QbD-type concepts to stress testing and its place in the development of stability-indicating analytical methods and stability-control strategies. Knowledge space, design space, and control space scenarios are summarized.

reactions are not included in this list (as they are not primarily driven by temperature), but are discussed separately in more detail (see below). The ICH Stability guideline suggests studying "... the effect of temperatures in 10°C increments above the accelerated temperature test condition (e.g., 50°C, 60°C, etc.)" It is not clear why the guideline suggests 10°C increments, but it may be related to the importance of understanding whether or not any degradation mechanisms (especially in the solid state) change as a result of increasing temperature. Studies with such incremental temperature increases would be useful for constructing Arrhenius plots to enable the prediction of degradation in the solid-state rates at different temperatures. However, for many pharmaceutical small molecule drug substances, it would take several months or more of storage at the elevated temperatures to induce enough degradation to provide meaningful kinetic data from which to construct such plots.

As discussed in chapter 1 (see section "Historical Context"), the kinetics of drug degradation has been the topic of numerous books and articles. The Arrhenius relationship is probably the most commonly used expression for evaluating the relationship between rates of reaction and temperature for a given order of reaction (for a more thorough treatment of the Arrhenius equation and prediction of chemical stability, see Refs. 1–3, 36–38). If the decomposition of a drug obeys the Arrhenius relationship [i.e., $k = A \exp(-E_a/RT)$, where k is the degree of rate constant, A is the pre-exponential factor or frequency factor (i.e., the frequency of collisions among reactants irrespective of energy), R is the universal gas constant, and T is the temperature in degrees kelvin], it is possible to estimate the effect of temperature on the degradation rate of a compound, providing the energy of activation (E_a) is known (39).

Connors et al. (37) assert that most drug substances have energies of activation (E_a 's) of 12–24 kcal/mol, although E_a 's >24 kcal/mol are not uncommon (40). In 1964, Kennon (41)

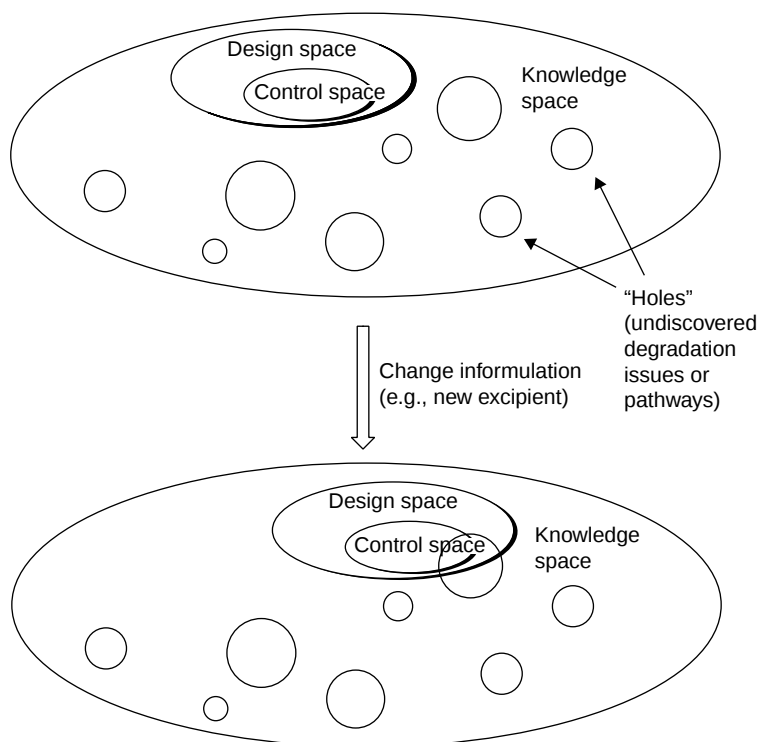


Figure 5 Conceptual representation of a change in the "location" of design and control spaces on the knowledge space surface, resulting in a design and control space that now has "holes," or unexpected/undiscovered degradation-related issues.

compiled the activation energies for decomposition of a number of drug compounds and found the average to be 19.8 kcal/mol. As noted by Zahn in chapter 23, the USP has recommended the assumption of E_a as ~19.87 kcal/mol (83.14 kJ/mol) for mean kinetic temperature calculations (42) Davis (43,44), a retired FDA reviewer, has asserted that 20 kcal/mol is a quite conservative estimate for the average E_a of decomposition of drug compounds. The "Joel Davis Rule," a historical rule-of-thumb that has been commonly used in the pharmaceutical industry, states that acceptable results from 3-month stability testing of a drug product at 37–40°C can be used to project a tentative expiry date of 2 years from the date of manufacture at 25°C/60% RH. Yang and Roy have shown that this rule-of-thumb is valid only if the E_a is >25.8 kcal/mol. The PhRMA "Available guidance and best practices" on forced degradation (27) and Alsante et al. (18) have recommended a conservative approach of assuming that for every 10°C increase in temperature the reaction rate approximately doubles. This is approximately equivalent to assuming an E_a of 12 kcal/mol.

More recent work has revealed that such a low estimate (E_a of 12 kcal/mol) is extremely conservative. MacFaul et al. studied the kinetics of degradation of 166 drug-like compounds in *solution* at elevated temperatures (46). These studies showed that the mean E_a was 23.6 kcal/mol (98.6 kJ/mol), with a range of 11.9 kcal/mol to 47.2 kcal/mol. Data from *solid-state* degradation studies of more than 50 compounds in 100 studies at Pfizer using the ASAP approach indicated an average E_a of 29.8 kcal/mol (124.7 kJ/mol) (38); similar results have also been obtained in a more limited dataset (nine compounds in 20 studies) at Lilly (47).

The approach of predicting shelf-life time frames solely on activation energies, to enable the modeling of rates of degradation in the solid state, is flawed in that it does not take into consideration the relative humidity. Waterman has, however, outlined a short-term approach (e.g., 2–3 weeks) for developing a predictive model for chemical stability in the solid state using a modified Arrhenius approach (1–3,38,48) that takes into account the relative humidity. This approach, known as the "Accelerated Stability Assessment Program" (ASAP), involves stressing at different temperatures, humidities, and times, with a goal of inducing the same amount of degradation at all conditions (an "isoconversion" approach). Using the ASAP approach, it has been demonstrated that degradation rates of formulated products (with pathways involving either hydrolytic or oxidative degradation or both) will usually hold to the Arrhenius relationship if (i) the relative humidity is held constant at the different elevated temperatures or (ii) relative humidity is built into the kinetic model. ASAP therefore incorporates relative humidity into a modified Arrhenius relationship. (1–3,38,48). Thus, a kinetic model can be constructed to allow prediction of rates of degradation at different temperatures and humidities. Continuing research in this area by researchers at Pfizer (38,48) and Lilly (47) provide strong support for the validity of this modified Arrhenius approach.

Tables 1 and 2 show rates of degradation relative to 25°C assuming energies of activation of 12 to 29.8 kcal/mol assuming that the degradation follows Arrhenius kinetics. Table 1 can be used to estimate the effect of stress temperatures on the rate of a degradation reaction for a particular E_a . It is apparent from Table 1 that the increase in reaction rate is dependent on the E_a , and that a low energy of activation (e.g., 12 kcal/mol) results in a less-dramatic increase in reaction rate as temperature is increased.

Using the information provided in Table 1, it is straightforward to calculate the effect of temperature on the degradation rate to enable prediction/estimation of degradation rates at lower temperatures (e.g., room temperature) for different energies of activation. For example, if one assumes an activation energy of 12 kcal/mol, stressing at 70°C for 1 week would be roughly the same as 100 days at 25°C ($14.3 \times 7 \text{ days} = 100.1 \text{ days}$). Similarly, assuming an activation energy of 19.8 kcal/mol and stressing at 70°C for 1 week would be roughly the same as 14 years at 25°C ($739.8 \times 7 \text{ days} = 5179 \text{ days or } 14.1 \text{ years!}$).

It should be noted here that solid-state reactions often proceed in an "autocatalytic" pathway (similar to oxidative degradation kinetics) involving an induction period (lag), followed by a period of rapidly increasing degradation and then a slowing down of the degradation rate

as the drug is consumed (50,51). Thus, solid-state reaction kinetics will often follow an S-shaped curve when degradation versus time is plotted. This kind of reaction kinetics is often more pronounced in formulated solid oral dosage forms (for reasons which will not be discussed here). It is reasonable to question whether or not Arrhenius kinetics will hold if the solid-state degradation is autocatalytic. In fact, Arrhenius kinetics are typically observed in the degradation of solid pharmaceutical products (within temperature ranges discussed in what follows) presumably because most solid-state degradation studies involve only modest amounts of degradation (e.g., ~5%) and are therefore typically operating in the “induction” or “lag” period of the solid-state degradation. If solid-state degradation studies are carried out to higher levels of degradation (e.g., >10–30% degradation), it is likely that degradation rate prediction via Arrhenius kinetics would not be accurate.

Note that the above information assumes that the decomposition follows the same pathways at all the temperatures. This assumption will not be true for all compounds, but for the majority of small molecule drug compounds; it is our experience that for either solid-state or solution-state studies, the degradation pathways will usually be the same up to ~70°C, assuming that there are no significant phase changes across the temperature range [e.g., melting points, deliquescence (52,53), salt disproportionation/change in ionization state, (54) etc.]. Precedence can be found in regulatory guidelines and in the scientific literature for using temperatures up to 50°C (4), 60°C (7), and even 80°C (55) and 85°C (41,56) for stress testing and “accelerated stability” studies. However, the references to stressing at 80°C and 85°C suggest that such high temperatures are optional and may lead to different decomposition pathways for some compounds. MacFaul et al. described the use of stress-testing temperatures as high as 90°C in solution, with no significant deviations from Arrhenius kinetics; interestingly, in a number of cases the degradation profile at higher temperatures was significantly different than at lower temperatures (yet the degradation still followed Arrhenius kinetics) (46). In a different

Table 1 Rates of Degradation (Relative to 25°C) Assuming Arrhenius Kinetics and Energies of Activation (E_a) of 12, 17, 19.8, 25.8, and 29.8 kcal/mol

Temperature (°C)	Relative Rate ^a				
	E_a = 12 kcal/mol (50.2 kJ/mol)	E_a = 17 kcal/mol (71 kJ/mol) (49)	E_a = 19.8 kcal/mol (82.8 kJ/mol) (41,42)	E_a = 25.8 kcal/mol (107.8 kJ/mol) (43,44)	E_a = 29.8 kcal/mol (124.6 kJ/mol) (38)
25	1	1	1	1	1
30	1.4	1.6	1.7	2.1	2.3
40	2.6	4.0	5.0	8.1	11.2
50	4.8	9.2	13.3	29.2	49.3
60	8.4	20.4	33.7	97.7	198.9
70	14.3	43.2	80.6	304.8	739.8
80	23.6	86.6	183.6	891.2	2554.7

^aThe relative rate is meaningful only within individual columns. Relative rates across rows should not be inferred.

Table 2 Calculated Number of Days to Simulate 24 Months Storage at 25°C using Arrhenius Kinetics and Different Energies of Activation

Temperature (°C)	E_a = 12 (kcal/mol)	E_a = 17 (kcal/mol)	E_a = 19.8 (kcal/mol)	E_a = 25.8 (kcal/mol)	E_a = 29.8 (kcal/mol)
40	280.8	182.5	146.0	90.1	65.2
50	152.1	79.3	54.9	25.0	14.8
60	86.9	35.8	21.7	7.5	3.7
70	51.0	16.9	9.1	2.4	1.0

study, an example of changes in degradation mechanism above 80°C is illustrated by the case of SB-243213 (57). In this example, stress studies in the solid state at room temperature up to 80°C showed the same degradation profile. In contrast, stressing at 100°C showed a large number of new degradants not observed at lower temperatures. Another example of changes in degradation mechanism as a function of temperature can be seen in the case of cefaclor.

Cefaclor is an oral cephalosporin antibiotic whose degradation pathways have been studied extensively (58). As shown by Dorman et al. (59), the degradation profile of cefaclor monohydrate after storage at 85°C in the solid state is significantly different than the profile observed upon storage at room temperature or at 40°C. Olsen et al. (60) have shown that the degradation pathways of cefaclor at room temperature do not change as the temperature is increased to ~70°C (see also Fig. 2 in chap. 18). Somewhere between 70°C and 85°C, different degradation pathways begin to occur, illustrating that there is a risk of introducing irrelevant degradation pathways, as stressing temperatures of drug substances and products are increased.

How Long?

With a maximum temperature in mind, the next question to answer is “How long should the sample be stressed?” The PhRMA guidance (27) and a draft PhRMA white paper (61) provides a recommendation that has been embraced by much of the industry, that is, the solid-state thermal/humidity stress test should be equal or greater than the “kinetic equivalence” of 6 months at 40°C/75% relative humidity. Thus, the thermal energy imparted to the sample experiencing thermal stress over the course of the study should be equivalent to or greater than that imparted to a sample in an accelerated study at 40°C over 6 months, with consideration of the humidity experienced by the sample. Determination of the true thermal kinetic equivalent requires either knowledge of the E_a or use of an assumed E_a . If one assumes a conservative 17 kcal/mol E_a , the thermal energy equivalent to 6 months at 40°C is 2 years at 25°C (assuming the same relative humidity); if one assumes a less conservative E_a such as 25.8 kcal/mol, the thermal energy is equivalent to 4 years.

In conclusion, on the basis of evaluation of the literature and kinetic considerations in conjunction with our stress-testing experience over the last 20 years, Temperatures of up to 70°C at high (e.g., 75% or higher) and low (e.g., 0–20%) relative humidities should provide a rapid, reasonably predictive assessment of the solid-state degradation pathways and relative stabilities of most drug substances at lower temperatures. The time period should allow for a thermal kinetic equivalence at least 6 months at 40°C/75% RH. Assuming a conservative E_a of 17 kcal/mol, 17 days of stressing at 70°C will exceed the thermal equivalent of 6 months at 40°C/75% RH. Table 3 shows the overall recommendations for solid-state thermal/humidity stressing.

Hydrolytic Degradation

Drug degradation that involves reaction with water is called hydrolysis. Stewart and Tucker (63) have asserted that hydrolysis and oxidation are the two most common mechanisms of drug

Table 3 Thermal/Humidity Recommended Stress Conditions

Container	Open
Sample	Active pharmaceutical ingredient: Use representative synthetic route material and physical form Drug product: Use high and low potencies (if applicable) of definitive product
Temperature	50–70°C
Relative humidity (62)	High humidity: 75% or greater Low humidity: 20% or lower
Maximum duration	Achieve kinetic equivalent to 6 months at 40°C/75% RH or greater. See Tables 1 and 2 for guidance

degradation. The experience of the authors and extensive reviews of drug degradation literature are consistent with their assertion. Given that water is present at significant levels in many drugs (e.g., hydrates), in many excipients, and even at normal atmospheric conditions, it is not surprising that hydrolysis is a common degradation problem. Because hydrolysis is such a common reaction (it has been described in detail elsewhere), it will not be extensively dealt with in this chapter. Rather, just a few of the more important aspects will be discussed, with relevant literature references given to facilitate further study.

Stewart and Tucker assert that hydrolysis is affected by pH, buffer salts, ionic strength, solvent, and other additives such as complexing agents, surfactants, and excipients, and each of these factors is discussed in some detail. Waterman et al. (64) provide a focused discussion of hydrolysis as it relates to pharmaceuticals, with thorough discussions of mechanisms, formulation considerations, pH, ionic strength, buffers, solid-state considerations, hydrolysis of lyophiles, liquid dosage forms, packaging, etc. Mabey and Mill (65,66) provide a critical review of the hydrolysis of organic compounds in water and a thorough treatment of the topic, especially as it relates to environmental degradation.

Hydrolysis reactions are typically acid or base catalyzed. Acidic, neutral, and basic conditions should therefore be employed in order to induce all potential hydrolytic reactions. This is especially important when the compound being tested has ionizable functional groups and can exist in different ionization states under relevant aqueous conditions. It is particularly important to test hydrolysis at unique protonation states, unless there are a large number of ionizable functional groups, as is often the case with peptides and proteins. In cases such as these, a practical approach is to simply expose the sample to a wide pH range in defined increments (e.g., 1 pH unit). A pH range of 1 (e.g., 0.1 M HCl) to 13 (e.g., 0.1 M NaOH) has been used by a number of major pharmaceutical companies (15) for the most acidic and most basic extremes of aqueous stress testing. As discussed in the sections "Historical Context" and "Regulatory Context" in chapter 1, more acidic (e.g., $\text{pH} < 1$) and basic (e.g., $\text{pH} > 13$) conditions can and have been employed. These unusually acidic or basic conditions may simply speed up acid or base-catalyzed hydrolysis, but there is an increased risk of inducing unrealistic degradation pathways (e.g., from protonation of sites with very low pKs that can alter the site(s) of hydrolytic attack). Additionally, neutralization of the solution prior to HPLC analysis is not recommended due to the possibility of precipitation or secondary reaction artifacts.

One problem that is often faced in designing hydrolytic stress tests is compound solubility. Many small molecule drugs are not soluble in water at the concentrations typically used for analytical evaluation (i.e., 0.1–1 mg/mL) across the entire pH range (15). Thus, either a slurry/suspension must be used to examine the hydrolytic stability of a compound or a cosolvent must be added to facilitate dissolution under the conditions of low solubility. The two most commonly used cosolvents are acetonitrile and methanol (15). Because methanol has the potential of participating in the degradation chemistry (e.g., acting as a nucleophile to react with electrophilic sites or intermediates in the degradation pathways), it should be used with caution (especially under acidic conditions) if the compound being tested contains a carboxylic acid, ester, or amide as these groups may react with methanol. Acetonitrile is generally regarded as an inert solvent and is typically preferable to methanol in hydrolytic stress-testing studies (15). It should be recognized, however, that acetonitrile is not completely inert and can participate in the degradation reactions leading to artifactual degradation results. For example, acetonitrile is known to contribute to base-catalyzed epoxidation reactions in the presence of peroxides (67,68). Acetonitrile will also degrade, in the presence of bases (e.g., $\text{pH} 13$) and/or acids (e.g., $\text{pH} 1$) under elevated temperatures, to detectable levels of acetamide and/or acetic acid, which can show up as early eluting peaks (when monitoring with UV at low wavelengths) on RP-HPLC. The size of the HPLC peaks from these two products is relatively small, and the use of stressed blank solutions (solvent system without drug stressed under the same conditions) permits ready identification of these peaks. In acidic acetonitrile/water solutions, tertiary alcohols can undergo a Ritter reaction to form amides (see Fig. 91 in chap. 3). In the presence of radicals [e.g., generated during prolonged

sonication as part of the analytical work-up or in the presence of free radical initiators such as 2,2-azobisisobutyronitrile (AIBN)], acetonitrile can be oxidized to small amounts of formyl cyanide that will readily react with nucleophiles (such as amines), resulting in a formylation reaction (Fig. 6). Skibic et al. showed that titanium dioxide can catalyze the degradation of acetonitrile in the presence of either sonication or light, resulting ultimately in the artifactual formylation of a secondary amine (69). Nonetheless, most of these side reactions of acetonitrile are relatively minor and acetonitrile remains the most frequently used cosolvent for hydrolysis studies. Other cosolvents that have been recommended for hydrolytic stress-testing studies (70) are shown in Table 4.

The potential effects of cosolvents on the degradation rates and pathways are worth discussing. It is often thought that the apparent hydrolytic degradation rate of a drug will be increased by the use of a cosolvent to facilitate dissolution; however, this is not always the case. (In our experience, roughly 25–40% of the time the observed degradation rate in aqueous conditions will be slower with the addition of a cosolvent such as ACN when compared with an aqueous slurry/suspension.) The overall hydrolytic degradation rate will depend on the specific mechanism(s) involved in the degradation pathway(s). The degradation reactions and rates involved will depend on a variety of factors such as the dielectric constant, solvent polarity, ionic strength, whether or not the solvent is protic or aprotic, the surface energy (i.e., of the solid–liquid interface in a slurry/suspension), etc. (71–78). For example, a degradation reaction involving acid-catalyzed hydrolysis with a cationic intermediate or a polarized transition state will be facilitated by a solvent with a high dielectric constant, and the addition of a cosolvent that reduces the effective dielectric constant will reduce the rate of such a reaction.

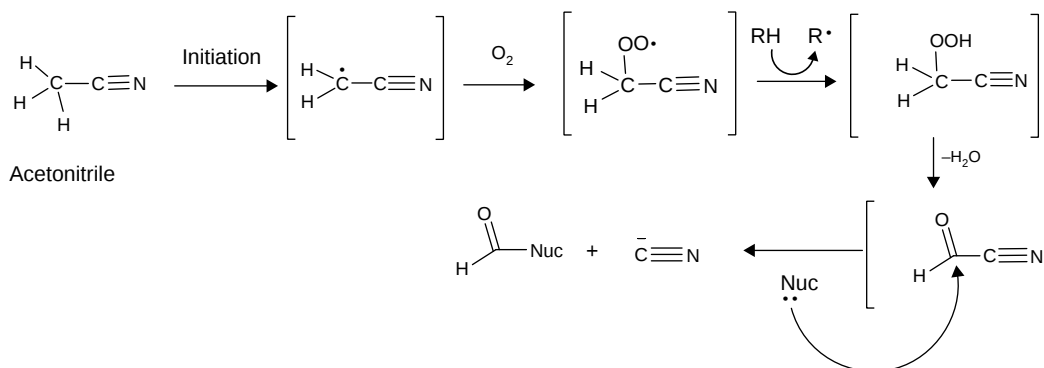


Figure 6 Potential side reaction of acetonitrile in the presence of radicals. As described in the text, an example of this reaction has been documented in the literature, where the reaction is catalyzed by titanium dioxide (often present as an ingredient in drug product colorants) when exposed to either light or sonication (69). (Nuc = nucleophile).

Table 4 Organic Cosolvents that Have Been Used for Stress-Testing Studies

Acidic pH	Neutral pH	Basic pH
Acetonitrile ^a	Acetonitrile ^a	Acetonitrile ^a
DMSO	<i>N</i> -methylpyrrolidone (NMP) (for oxidizing conditions primarily)	DMSO
Acetic acid	Methanol	Glyme ^a
Propionic acid		Diglyme
THF		<i>p</i> -Dioxane
		Methanol

^aVolatile solvent—may evaporate at higher temperatures.

Solvation of a compound in an aqueous cosolvent mixture may involve formation of a “solvent cage” of the more nonpolar solvent around the compound, potentially leading to some protection from hydrolysis. Solvent composition can also affect tautomeric states of molecules (79,80), which in turn can affect both degradation rates and pathways. The effective pH of an aqueous solution will also change upon addition of a cosolvent (81–83), which can both affect the degradation rate and change the degradation pathway(s) (e.g., by facilitating different protonation states).

The use of elevated temperature is appropriate (though not required) for aqueous solution stress-testing studies; the use of appropriate thermal controls is recommended. As discussed in the section “Thermolytic Degradation,” elevated temperatures up to 70°C should accelerate the hydrolytic degradation processes in a meaningful way. Higher temperatures can be used, but the risk of non-Arrhenius behavior increases significantly when temperature is increased further. As discussed earlier in the chapter, MacFaul et al. observed Arrhenius behavior for 166 compounds acid/base solution studies at temperatures up to 90°C, but the degradation profiles sometimes changed at the higher temperatures, illustrating the risk. Tables 1 and 2 can be used to help predict degradation rates at different temperatures, with the assumptions of Arrhenius behavior and specific activation energies.

In conclusion, we assert that testing of the hydrolytic susceptibility of a drug substance should involve exposure to acidic, neutral, and basic conditions in the pH range of 1–13, preferably, but not necessarily, under 100% aqueous conditions. When solubility is low, the use of an inert water miscible cosolvent (e.g., acetonitrile or other solvents shown in Tables 4 and 5) is appropriate, but it should be recognized that the presence of a cosolvent may either speed up or slow down the hydrolysis, and there is a possibility that degradation pathways could also change in the presence of a cosolvent. Therefore, it may be useful, but not required, to stress the compound as a slurry in a 100% aqueous condition in addition to stressing in the presence of a cosolvent. Elevated temperatures with an upper limit of 70°C are recommended for accelerating the hydrolytic reactions. The longest recommended time period for stressing at the highest temperature is 1 week, although longer times can certainly be used if desired. The author recommendation for acid and base catalyzed hydrolytic forced stress studies are summarized in Tables 6 [active pharmaceutical ingredient (API)] and 7 (drug product).

Oxidative Degradation

Oxidative reactions are one of the two most common mechanisms of drug degradation. There are three major oxidative pathways important to consider for drug degradation: (i) radical-initiated oxidation (also known as autoxidation); (ii) peroxide-mediated oxidation; (iii) electron-transfer mediated oxidation. Stress-testing studies designed to mimic these pathways are discussed here as “predictive” oxidative stress tests. Oxidative pathways from exposure to other oxidizing conditions, for example other reactive oxygen species such as hydroxyl radicals, singlet oxygen, or ozone are possible, but less common; such tests will be discussed here as “investigative” oxidative stress tests.

Predictive Oxidative Stress Tests

Radical-Initiated Oxidation (Autoxidation)

Autoxidation is typically thought of as a radical-initiated process, although the source of initiation may or may not be well understood, depending on the system (typically light induced, metal catalyzed, homolytic bond breakage of peroxides, etc). Radical-initiated reactions start with an initiation phase involving the formation of radicals (this step is rate limiting), followed by a propagation phase and eventually a termination phase. Thus, the reaction kinetics will often follow an S-shaped curve when degradation versus time is plotted (50,51,84) and may not follow Arrhenius kinetics. In the solid state, it is not clear whether or not oxidative degradation reactions have significant propagation phases. It has been recently suggested that autoxidative degradation reactions in solid oral dosage forms do not have significant propagation phases

Table 5 Cosolvent Selection Guide

Cosolvent	Pros	Cons
DMSO	Good general solvent, generally inert toward drugs, completely miscible in water, can be removed by lyophilization but otherwise nonvolatile. Useful under acidic and basic conditions.	Absorbs in low UV region, shows up as UV peak in chromatogram.
Acetic acid	Completely miscible in water. Useful under acidic conditions.	May react with some alcohols to form esters and low pK_a amines to form amides, peak in UV at low wavelengths.
Propionic acid	Good cosolvent for steroids. Useful under acidic conditions.	May react with some alcohols to form esters and low pK_a amines to form amides, peak in UV at short wavelengths, not soluble in water in all proportions.
<i>N</i> -methyl-pyrrolidone (NMP)	Completely miscible in water, generally inert toward drugs, good medium for autoxidation, nonvolatile. Useful under natural and neutral pH conditions. Good model for drug interactions with PVP and povidone. Hydrolyzes very slowly under neutral conditions.	Peak in UV. May contain and/or will form peroxide impurities. Use only fresh solvent under an inert atmosphere if oxidation is not desired.
Acetonitrile (ACN)	Completely miscible in water. UV transparent. Cosolvent of choice for photochemistry. Good solubilizer for many compounds. Inert toward most active pharmaceutical ingredients. Can lyophilize aqueous solutions containing ACN.	Volatile. May lose solvent at higher temperatures unless well sealed. Hydrolyzes in acid/base to form acetic acid or acetamide. Above neutral pH in oxidizing conditions can produce reactive per-acid like compounds. Can be oxidized by alkoxyl radicals resulting in formylation of amines.
Glyme	Completely miscible in water, inert to base, UV transparent. Useful under basic conditions. Oxidizes slowly in base.	Too volatile. Rapidly oxidizes under neutral and acidic conditions.
Diglyme	Completely miscible in water, inert to base, UV transparent. Useful under basic conditions. Oxidizes slowly in base. Better than Glyme for most applications.	Rapidly oxidizes under neutral and acidic conditions.
<i>p</i> -Dioxane	Completely miscible in water, inert to base. UV transparent. Good medium for autoxidation.	Volatile. Rapidly oxidizes under acidic or neutral conditions.
Tetrahydrofuran	Completely miscible in water; inert to base. UV transparent.	Volatile. Rapidly oxidizes under most conditions. May contain stabilizers that complicate chromatography.
Methanol	Completely miscible in water. UV transparent. Good solubilizer for many compounds. Good scavenger of hydroxyl radicals at concentrations of 10% or higher.	Reactive toward some functional groups (esp. esters or carboxylic acids), especially in acid. May contain trace formaldehyde.

(presumably due to lack of mobility), and therefore, observed deviations from Arrhenius kinetics may be from different causes (85). The picture is further complicated by the complex nature of oxidative reactions, where oxidative intermediates are often thermally unstable and may decompose via alternate pathways at elevated temperatures (86). Increases in temperature,

Table 6 Acid and Base Degradation Recommended Conditions (API)

API concentration	0.1–1 mg/mL
pH ~1 and ~13	0.1 M HCl and 0.1 M KOH or NaOH
pH 2–12	Phosphate buffer, 50 mM, pH adjustment with HCl or NaOH/KOH, as needed.
Cosolvents	Acetonitrile (or use Table 5 as a guide.)
Temperature	Room temperature to 70°C
Duration	5–20% degradation up to 1 week at 70°C
Neutralization	Not recommended: high risk of artifacts, secondary reactions, etc.
Containers	Flint or borosilicate glass vials with airtight closures to minimize solvent evaporation.

Table 7 Acid and Base Degradation Conditions (Drug Product)

API concentration	Formulation dependent
pH range	+/- 2 pH units around the target pH
Temperature	Up to 70°C
Duration	5–20% degradation or 1–3 weeks maximum, depending on shelf-life needs.

Table 8 Recommended Autoxidative Screening Conditions

Initiator	Azonitrile such as AIBN ^a (organic soluble) or ACVA ^b (water soluble)
API concentration	0.1–1 mg/mL
Initiator concentration	5–20 mol% of API concentration
Solvent	Acetonitrile/water/methanol ^{a,b}
Temperature	40–60°C recommended for AIBN and ACVA
Duration	5–20% degradation or 7 days maximum

^aAIBN = azobisisobutyronitrile, also known as VAZO 64. Recommended solvent system for this azonitrile is ACN (50% or greater)/MeOH (10%)/water (0–25%).

^bACVA = 4,4'-azo-bis(4-cyanovaleric acid. Recommended solvent system for this azonitrile is water (50% or greater)/MeOH (10%)/ACN (<40%).

therefore, may not lead to predictable changes in degradation rates, and the observed oxidative rates and pathways may be different than those observed at lower temperatures. Nonetheless, as discussed previously in this chapter, if the effects of relative humidity are taken into account, Arrhenius kinetics are often followed for solid oral dosage forms when comparing rates across temperature ranges in which there are no phase changes (1–3,48). In solution, oxidative rates and pathways may be dependent on the dissolved oxygen concentration. Thus, the reaction rate in solution may actually be reduced at higher temperatures because of the decrease in oxygen content of the solvent. It is assumed the reaction of the radical with oxygen to form a peroxy radical occurs at the diffusion controlled rate of $10^9 \text{ mol}^{-1}\text{s}^{-1}$ at room temperature/atmospheric pressure. This may be partially overcome by bubbling oxygen or air through the solution while heating or by storing the solution under oxygen in an airtight vessel with high pressure (at least a few atmospheres). Regardless, in solution, the degradation kinetics will likely not follow Arrhenius kinetics, and therefore short-term stress studies may not accurately predict long-term stability in solution. Instead, the general susceptibility of a compound to autoxidative degradation (and the resulting oxidative degradation products formed) can be studied in solution using a radical initiator [e.g., AIBN (azobisisobutyronitrile) or ACVA (4,4'-azo-bis(4-cyanovaleric acid))] (87). Table 8 shows some recommended conditions for carrying out such a test. It is worth noting here that methanol is recommended as part of the solvent

Table 9 Recommended Conditions for Oxidative Stressing with Hydrogen Peroxide

Hydrogen peroxide	Reagent concentration	0.3–3%
	API concentration	0.1–1 mg/mL
	Temperature	Room temperature to 30°C
	Duration	5–20% degradation or 7 days maximum

Table 10 Recommended Conditions for Oxidative Stressing with Transition Metals

Transition metals Note: Samples should be prepared such that oxygen is available (i.e., dissolved oxygen should be present in solution)	Metal	Cu(II) (e.g., CuCl ₂ or CuSO ₄) Fe(III) (e.g., FeCl ₃ or Fe ₂ (SO ₄) ₃)
	Concentration	1 mM
	API concentration	0.1–1 mg/mL
	Temperature	30–40°C
	Duration	5–20% degradation or 1 day maximum

system at a concentration of 10%; this is due to the ability of methanol to act as a hydrogen atom donor and scavenge any alkoxyl radical that might form during the radical-initiator test. Alsante et al. have also found that increasing the mol% initiator to 10 mol% is a more efficient way to increase % radical reactions as opposed to increasing oxygen pressure. For a more thorough discussion of this topic and oxidative stress testing and recommended conditions in general, including additional azonitrile options, see chapter 6.

Peroxide-Mediated Oxidation

Oxidation of pharmaceuticals can also be caused by exposure to peroxides. Peroxides can be found in varying levels in certain excipients (88) (e.g., polysorbates, polyethylene glycol, polysorbates, povidone, and hydroxypropyl cellulose) and the levels may increase over time due to autoxidation processes. Thus, it is common for drug substances to be exposed to peroxides after formulation and upon storage and distribution. The classic oxidative stress test is to expose drug substances to peroxides by dissolving the drug in dilute aqueous hydrogen peroxide (e.g., 0.3–3% hydrogen peroxide). It may be useful to control the pH to ensure that all relevant protonation states of the drug are tested. For example, if the compound being tested contained one ionizable functional group (e.g., an amine) with a pK of 7, the oxidative tests could be carried out 1 pH unit above and below. It should be noted here that room temperature storage is sufficient for the hydrogen peroxide test. The use of higher temperatures (e.g., >30°C) with hydrogen peroxide should be done with caution because the O–O bond is a weak bond that will cleave at elevated temperatures to form hydroxyl radicals, a much harsher oxidative reagent, that will aggressively oxidize most drugs by unrealistic or nonpredictive pathways. Instead, it is recommended to do the peroxide exposure at ≤ 30°C in the dark for 1–7 days. Such conditions allow the nonradical peroxide oxidative mechanisms to dominate (e.g., electrophilic attack and nucleophilic additions), permitting more realistic predictive assessments of degradation caused by peroxides. See Table 9 for recommended conditions for peroxide-mediated oxidative stress testing. For a thorough discussion of this topic, see chapter 6.

Electron-Transfer Mediated Oxidation

Electron transfer mechanisms (e.g., those involving removal of an electron from a functional group within a drug molecule) can occur as a result of binding to and reaction with metals such as copper(II) and iron(III). Upon the gain of an electron from the drug, the oxidation state of the metal is reduced and the drug molecule is oxidized to an unstable radical cation, readily reacting with molecular oxygen to form oxidative degradation products. The use of transition metals (e.g., copper(II) and iron(III) at ~1–5 mM, 1 day) is also recommended for evaluation of oxidative susceptibility. See Table 10 for a summary of the recommended conditions. For a

more thorough discussion on oxidative degradation and stress testing, see Refs. 86, 89, 90, 91, and chapter 6.

Investigative Oxidative Stress Tests

As mentioned above, oxidative degradation can be very complex, and it can be useful to have in one's "tool box" other oxidative reagents/techniques to allow for rapid investigation of oxidative degradation problems including isolation or enhanced reaction mixtures for structure elucidation efforts. For example, the use of peroxides stronger than hydrogen peroxide (e.g., peracetic acid, meta-chloroperoxybenzoic acid (mCPBA), potassium monoperoxysulfate (Oxone®) can often yield significant levels of oxidative products in less than 30 minutes.

Other useful oxidative reagents include singlet oxygen, sodium hypochloride ("bleach"), potassium permanganate (KMnO_4), and *N*-methyl pyrrolidone (NMP). A simple system to produce singlet oxygen involves the use of photosensitizers such as Rose Bengal or methylene blue in the presence of visible light, again for less than 30 minutes (91). Singlet oxygen will often produce some of the same products formed by other oxidative mechanisms, and thus can be useful for producing larger quantities of degradation products for identification purposes. Further, singlet oxygen-derived degradation, while unusual, is not unprecedented (92). Sodium hypochlorite (NaOCl) has been used to study oxidative degradation of drugs (93,94) and amino acids, (95–97) and for comparison with one-electron oxidative pathways in vivo. Potassium permanganate has been used in stress-testing studies as a tool to selectively make certain known oxidative degradation products, complementary to hydrogen peroxide (98). Another useful oxidative reagent is Fenton's reagent (Fe^{2+} in the presence of hydrogen peroxide to form hydroxyl radical, a very aggressive oxidant). Fenton's reagent can in some cases be useful to rapidly produce certain oxidative degradation products in a matter of seconds (99). Table 11 shows recommended conditions for using various oxidative reagents as stress-testing investigative tools.

Photolytic Degradation

Photolytic degradation (as it applies to pharmaceutical stability) is the degradation that results from exposure to ultraviolet or visible light in the wavelength range of approximately 300–800 nm. Exposure to radiation at wavelengths <300 nm is not needed because a pharmaceutical compound would not experience such exposure during its life cycle. The first law of photochemistry, also known as the Grotthus–Draper Law (first proposed in 1817) states, "only radiation that is absorbed by a molecule can be effective in producing chemical changes in the molecule." Thus, for photolytic degradation to occur, radiation must be absorbed—either by the drug substance or by the formulation. Photodegradation rates are therefore directly dependent on the amount of incident radiation and on the amount of radiation that is absorbed by the compound or the formulation. It is important to remember that a compound may undergo photolytic degradation even if it does not itself absorb radiation in the UVA or visible region. This can only happen if there is some additional agent in the formulation, intentionally (excipients) or adventitiously present (impurities), that facilitates absorption. Reed et al. (100) have documented a classic example of this phenomenon, describing a phenyl ether-based drug substance that exhibited photodegradation with both UVA and visible light exposure even though the drug molecule itself did not absorb at wavelengths >300 nm. In this case, the presence of low levels of iron(III) was found to chelate with carboxylates present in the formulation (citrate), leading to a complex that absorbed in the UVA and visible regions. The proposed mechanism for photodegradation was the formation of hydroxyl radicals (via the photo-Fenton reaction), which caused oxidative degradation of the drug.

It is important to remember that the ICH photostability guideline (Q1B) refers to both forced degradation studies (stress testing) and confirmatory testing (101). As noted by Thatcher et al. (102) confirmatory photostability testing is designed to be a part of the definitive, formal stability testing, and can be thought of as being analogous to accelerated stability testing (which

Table 11 Recommended Conditions for Various Oxidative Reagents as Investigative Stress-Testing Tools

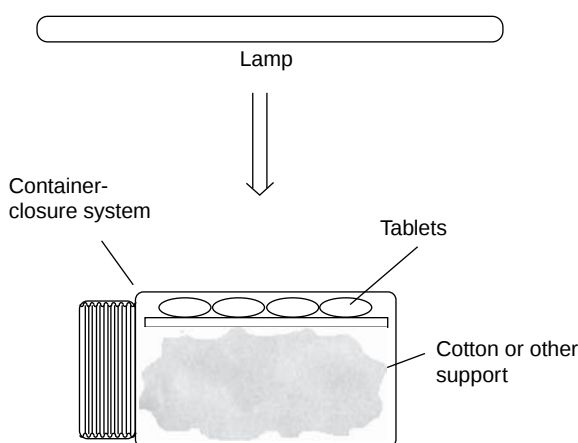
Fenton conditions (production of hydroxyl radicals)	Metal/concentration	Fe(II), e.g., FeCl ₂ , 1 mM
	Peroxide concentration	0.03–0.3%
	API concentration	0.1–20 mg/mL
	Temperature	0°C to room temperature
Singlet oxygen	Duration	5–20% degradation or 1hr maximum
	Photosensitizer	Rose bengal
	Photosensitizer concentration	0.1 mM
	API concentration	0.1–20 mg/mL
	Light source	Cool white fluorescent (1000–20,000 lux) or ICH Q1B Option 1 light source (e.g., xenon)
Peroxides	Duration	5–60 min (typically)
	Peracid	(a) Peracetic acid, 0.1–1 molar equivalent (b) <i>m</i> -Chloroperoxybenzoic acid (mCPBA), 0.1–1 molar equivalent (c) Potassium peroxymonosulfate (Oxone®), 0.1–1 molar equivalent
	Concentration	Variable. 0.1–1 molar equivalent, suggested
	API concentration	Variable. 0.1–1 mg/mL, suggested
Sodium hypochlorite (NaOCl, bleach)	Temperature	Room temperature to 40°C
	Duration	5 min to 1 day
	Reagent concentration	25–50 mM
	API Concentration	Variable. 0.1–1 mg/mL, suggested
	Temperature	Room temperature to 40°C
	Duration	5 min to several hours
Potassium permanganate	Solvent	Aqueous (organic cosolvent if needed)
	Reagent concentration	20 mM
	API concentration	Variable. 0.1–1 mg/mL, suggested
	Temperature	Room temperature to 40°C
	Solvent	ACN/water mixtures

is also part of the ICH Q1A definitive, formal stability testing for a drug). Thus, the minimum recommended exposure outlined in Q1B (i.e., 1.2 million lux-hr visible and 200 W h/m² UVA) is not the exposure recommended for forced degradation studies. In fact, there is no mention of recommended exposures for forced degradation studies and the design is left open. A member of the original ICH Photostability Expert Working Group recommended an exposure of three to five times the minimum ICH confirmatory exposure for forced degradation studies (103). Interestingly, early versions of the guideline (during step 1 of the ICH process) suggested that forced degradation studies should use exposures in the range of five to ten times the confirmatory exposure recommendations. A photoexposure in the range of two to five times the confirmatory exposure seems a reasonable amount of photostress for forced degradation studies, remembering that photodegradation of the compound being studied beyond 10–20% would not be necessary or desired.

It should be remembered that photodegradation products formed under stress conditions (i.e., potential photodegradation products) may not always be observed under confirmatory conditions, depending on such factors as the physical form of the compound (crystalline or amorphous solid, polymorphic form), if in solution the concentration and solvent, the protonation state and salt form, and other physical properties (particle size, surface area, crystallinity, etc.). Such differences may be exacerbated by the use of different photon sources

Table 12 Guidance for Conducting Photolytic Stress Testing

Visible light exposure	At least 2× the ICH confirmatory recommendation of 1.2 million lux-hrs
UV light exposure	At least 2× the ICH confirmatory recommendation of 200 W hrs/m ²
API in solution	Optional for solid dosage forms; recommended for IV, suspensions, and other liquid dosage forms
API solid state	API should be in a thin layer (i.e., <1 mm in depth)
Drug product in solid state	Tablets should be in a monolayer, as shown in the illustration in Figure 7
Light source	ICH Option 1 or 2 (consider using same source for stress and confirmatory testing)
Dark control	Cover container with light impermeable covering such as aluminum foil
Container	Quartz or borosilicate glass container with known light transmittance
Duration	Dependent on radiation intensity

**Figure 7** Schematic illustration of tablets arranged in a monolayer within a bottle for presentation to the light source. *Source:* From Ref. 104.

[i.e., ICH Option 1 vs. Option 2 (101)], irradiation intensities, exposure temperatures, and exposure times for stress testing and confirmatory studies, and therefore it may be prudent to use the same source for both studies to avoid confusion. (Different irradiation intensities and exposure temperatures can lead to differences in observed photodegradation profiles due to competing thermal reaction kinetics. For a discussion, see Ref. 104.)

Evaluation of the propensity of a drug substance (or formulation) to undergo photodegradation should be guided by the ICH QIB guidance document on photostability testing (101). As this topic has been covered extensively elsewhere (102–110), including chapters 7 and 8 in this book, it will not be discussed in detail here. For a summary of recommended conditions for photolytic stress testing, see Table 12.

INTRINSIC STABILITY: RATES OF DEGRADATION

A critical part of evaluation of intrinsic stability is the apparent rate of degradation under various conditions that lead to degradation. A rigorous evaluation of the kinetics of degradation is generally not the focus of stress-testing studies, but useful information can be gained by kinetic evaluation. For example, relative rates of degradation at different pH conditions (i.e., a pH-rate profile) allows an assessment of which pH conditions will provide the most (or least) stable environment for a compound. Such information is useful for evaluation of analytical sample preparation conditions (e.g., the sample solvent for analytical assay) and is critical to some solution formulations where stability must be maximized in order to achieve acceptable shelf

life. It can also be applicable in designing stable solid-state formulations. For example, if a compound is known to show instability under basic conditions, basic excipients that may cause an alkaline microenvironment in a formulation may be wisely avoided. Kinetics of degradation obtained from stress testing may also reveal the order of a reaction, the dependence of a solid-state degradation on humidity, or if the reaction is autocatalytic. Such information can be very useful in designing stability studies and interpreting early time point results. Kinetics of solid-state reactions have been thoroughly discussed elsewhere (for reviews of pharmaceutical kinetics see Refs. 1, 2, 3, 111, 112, and chap. 23) and will not be discussed further here.

In addition to following the kinetics of the disappearance of the parent compound, it can be very important in developing an understanding of the degradation pathways to follow the rates of formation of the different degradation products. Examination of the degradation profile from early time points (e.g., 0–5% degradation) can reveal which products are the primary or first formed products and which are secondary. This can be especially important, as it is not uncommon for the degradation profiles observed during real-time stability studies to contain a mixture of primary, secondary, and even tertiary degradation products in specific cases where the initial products are particularly unstable). Without kinetic time point analysis, one could miss unstable primary degradants and only observe secondary/tertiary degradants at elevated stress-testing conditions. For example, in the reaction schematic $A \rightarrow B \rightarrow C$, at room temperature, one could observe degradant B however, at elevated temperatures, one could miss B and only observe C. This would complicate predictive analysis if kinetic time points are not taken along the course of the reaction.

INTRINSIC STABILITY: STRUCTURES OF THE MAJOR DEGRADATION PRODUCTS

Knowledge of the structures of the major degradation products of a drug compound is a prerequisite to understanding the degradation pathways and, therefore, the intrinsic stability. Understanding the degradation pathways allows an assessment to be made of the sites in the compound that are susceptible to degradation under the different conditions. Such information is essential to an understanding of the intrinsic stability characteristics of a drug compound. The first critical issue is the definition of the major degradation products.

With regard to defining major degradation products, there is guidance available in the literature and a general consensus among some of the major pharmaceutical companies (61). Alsante et al. (18) have proposed a practical guide for defining major or key degradants; Table 13 shows the proposed criteria, which changes from preclinical to clinical and registration phases. The criteria are based on analytical evaluation of either the last stress time point for a given condition, or a time point that results in ~10–20% degradation of the parent compound. Thus, if the parent has degraded 10% in a particular sample from a certain stress condition, the major degradants would be defined as those that are greater in area than 10% of the total amount of degradation; for preclinical-phase 1 development, “major” is also defined as those peaks that are > 25% of the area of the largest individual degradant. Since relative response factors are often not known during preclinical and early development stages, it is reasonable to assume the same response factor (113) as the parent compound (unless there is good reason to suspect otherwise); such an assumption is consistent with ICH recommendations. This guidance by Alsante et al. is an effective algorithm for delineating major degradation products, which in the past has been more of an art, dependent on the judgment of the individual scientific researcher. A similar approach has recently been incorporated into a draft PhRMA white paper (61) as an algorithm to guide decisions on structure elucidation of degradation products that are discovered during stress-testing studies (i.e., a sort of stress-testing degradation product identification threshold), in the context of risk assessments for the potential formation of genotoxic degradation products. The draft PhRMA paper provides a rationale for the identification thresholds proposed, describing the intended connection to ICH Q3A and Q3B identification thresholds. The proposed thresholds are shown in Table 14 as a reference for the reader.

Table 13 Defining Major or Key Degradation Products from Stress Testing
[Recommendations by Alsante et al. (18)]

	Preclinical to Phase 2		Phase 2 to Registration	
	API	Drug Product	API	Drug Product
% of largest degradant	>25%	>10%	>10%	>10%
% of total degradation	>10%	>10%	>10%	>10%

Table 14 Proposed Thresholds for Identification of Degradants Formed During Stress-Testing Studies, Based on the Percentage of the Degradant Peak Area in a 1–10% Degraded Stress Sample (61)

Maximum Daily Dose (mg)	ID Threshold from ICH Q3B (%)	ID Threshold Derived from ICH Q3B for Stressed Samples Degraded 1–5% (%)	ID Threshold Derived from ICH Q3B for Stressed Samples Degraded >5–10% (%)	ID Threshold Derived from ICH Q3B for Stressed Samples Degraded >10–15% (%)	ID Threshold Derived from ICH Q3B for Stressed Samples Degraded >15–20% (%)
>2000	0.10	0.25	0.5	0.75	1.0
>10–2000	0.2	0.5	1.0	1.5	2.0
>1–10	0.5	1.25	2.5	3.75	5.0
<1	1.0	2.5	5.0	7.5	10.0

The second critical issue is whether or not degradation products arising from stress-testing studies should be identified at all. There appear to be two major schools of thought on this issue. One school of thought asserts that structure elucidation need only occur for those degradation products that are formed (in either the drug substance or the product) under long-term storage or accelerated testing stability studies at levels approaching or exceeding the ICH impurity threshold levels established in the relevant impurity guidelines (114,115). Using this line of thinking, major degradation products that occur during stress testing do not need to be identified unless they are also formed at significant levels during the shelf life of the product under long-term storage conditions (or possibly after 6 months storage under accelerated stability conditions, i.e., 40°C/75% relative humidity). Such an approach relies on the results of the analytical testing strategies and methods to resolve, detect, and accurately quantify the degradation products so that the major degradation products can be discerned. Assumptions have to be made regarding the response factors of the unknown degradants, the resolution from the parent compound, and the elution of the degradants from the column (assuming HPLC is the analytical technique). This strategy can be termed a “technique-oriented approach” (12,13) because it relies on the analytical technique to provide comprehensive and accurate results. The technique-oriented approach is strengthened by use of additional orthogonal analytical techniques (different HPLC columns and conditions, alternative separation techniques, different detectors, etc.). There are significant advantages to this approach, as there is a significant cost in resources and time to determine response factors and to elucidate chemical structures. Examination of part of the ICH definition of stress testing seems to support this approach:

However, it may not be necessary to examine specifically for certain degradation products if it has been demonstrated that they are not formed under accelerated or long term storage conditions.

Examining the sentence preceding the foregoing quote, however, reveals the context:

Examining degradation products under stress conditions is useful in establishing degradation pathways and developing and validating suitable analytical methods.

Thus, stress testing is, by definition, intended to help establish degradation pathways. One cannot establish a degradation pathway merely by identification of the conditions that lead to the degradation and noting the peaks that are observed in a chromatogram. Identification of a pathway involves both the conditions of degradation and the structures involved. It is the author's position that incomplete characterization of the degradation products is inconsistent with the principles of QbD, where scientific knowledge and thorough investigation provides the foundation for building quality into the product and the associated control strategies. Another problem with the technique-oriented approach is the reality that current analytical separation and detection technologies are not ideal as they cannot ensure resolution and quantification of unknown degradation products. When using HPLC with UV detection, the response factors of degradation products (relative to the parent compound) can be vastly different or even zero (e.g., nonchromophoric degradation products may not be detected with UV detectors at wavelengths >200 nm). In addition, since the degradation products are unknown (with unknown polarity, solubility, and volatility characteristics), it cannot be confirmed whether the degradation products will resolve from the parent, will elute from the column, or will even be amenable to the analytical technique. (For more discussion on this topic, see chaps. 4 and 9). Nonetheless, it can be *argued* that as long as the analytical methods result in the detection of degradation products (i.e., peaks in an HPLC–UV chromatogram), there are no clear regulatory requirements for structure elucidation of degradation products observed only during stress testing.

The other school of thought can be described as following a chemistry-guided approach (12,13). The chemistry-guided approach relies on scientific evaluation of the chemistry to guide the interpretation of the data and the selection of appropriate analytical techniques. An essential part of the chemistry-guided approach is developing an understanding of the structures of the major degradation products observed by the analytical method, which in turn allows an evaluation of the pathways, leading eventually to a rational assessment of the completeness of the investigation and the appropriateness of the analytical methodology.

An example of the use of the chemistry-guided approach can be seen in the case of LY297802 (Fig. 8) (12), described in chapter 9. In this example, degradation was observed upon exposure of a solution of the drug substance to cool white fluorescent light with no analytically observed (HPLC with UV detection) increases in degradation products. Additional analysis using another technique, LC/MS, revealed no additional information. Close examination of the container in which the degradation occurred, however, revealed a faintly observable insoluble film. The insoluble film was collected and analyzed by electron ionization (EI)-MS, revealing a prominent m/z of 256, with additional ions consistent with successive losses of 32 amu; this fingerprint EI-MS indicated that this material was elemental sulfur (S_8). This finding revealed that the photodegradation involved decomposition of the chromophoric part of the molecule (the thiadiazole ring). After unsuccessful attempts to detect these nonchromophoric degradation products by LC/MS, consideration was given to the possibility of the formation of volatile degradation products (which might not be detected by LC/MS). Analysis by GC/FID (and subsequently EI-MS) led to the identification of the other, previously undetected degradation products (i.e., *n*-butyl thiocyanate and the quinuclidine nitrile species, Fig. 8). This example serves to illustrate how the identification of a just a single degradation product (elemental sulfur in this case) can guide the analytical approaches used for a particular compound (chemistry-guided approach).

Another example of using the chemistry-guided approach is illustrated with the β -difluoronucleoside, gemcitabine hydrochloride (2'-deoxy-2',2'-difluorocytidine, Fig. 9) (116). Gemcitabine hydrochloride is currently marketed as a lyophilized powder; however, there was an interest in developing a solution formulation. Therefore, a study was designed to generate the data needed to construct an Arrhenius plot to enable prediction of degradation rates of solutions of gemcitabine at proposed storage conditions. The study solutions were also monitored for impurities resulting from the degradation of gemcitabine in order to compare the degradation profile of the investigational formulation with that of the marketed formulation.

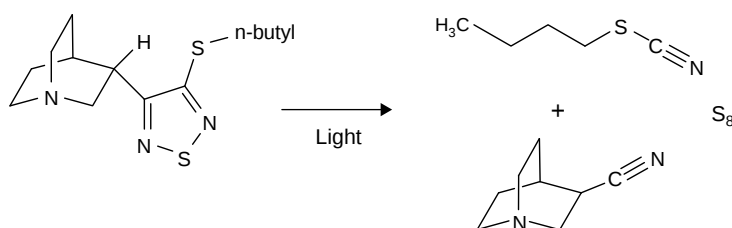


Figure 8 Structure of LY297802 and major photodegradation products.

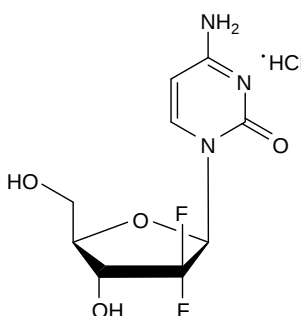


Figure 9 Structure of gemcitabine hydrochloride.

Table 15 HPLC Results Obtained on Thermally Stressed Solutions of Gemcitabine Hydrochloride

Condition	Gemcitabine Assay (% Initial)	Related Substances (%)	Mass Balance (%)	Relative Mass Balance Deficit ^a (%)
70°C/2 days	82.5	4.89	87.4	61.2
55°C/7 days	85.9	3.49	89.4	67.1
40°C/28 days	91.4	1.86	93.3	72.2
30°C/56 days	94.3	1.12	95.4	75.7

^aSee chapter 9 for a discussion of “relative mass balance deficit.”

During the course of the investigation, a significant mass balance issue was detected. Solutions of gemcitabine that exhibited a significant loss of the parent showed only a very minor increase in impurities. (Refer to Table 15 for selected results illustrating the mass balance issue and to Figure 10 for an HPLC chromatogram obtained on a solution thermally stressed at 70°C for 2 days.) Examination of the HPLC chromatogram (Fig. 10) indicates that the only significant impurity detected was the β -uridine analog (Fig. 11), a known degradation product of gemcitabine. The levels of the β -uridine analog were not high enough to account for the loss of the parent, indicating the formation of other degradation products that were not being detected.

A survey of the literature revealed a significant amount of information published on the degradation of cytidine, a structurally related nucleoside. Several papers (117,118) suggest a mechanism for deamination of cytidine to uridine that involves intermediates in which a nucleophile has been added to position 6 of the cytosine moiety, resulting in loss of the 5, 6-double bond. If the analogous chemistry were to occur with gemcitabine, the intermediates formed would likely have a significantly different UV spectrum than gemcitabine and might not be detected at the wavelength used in the HPLC method (275 nm). The wavelength was

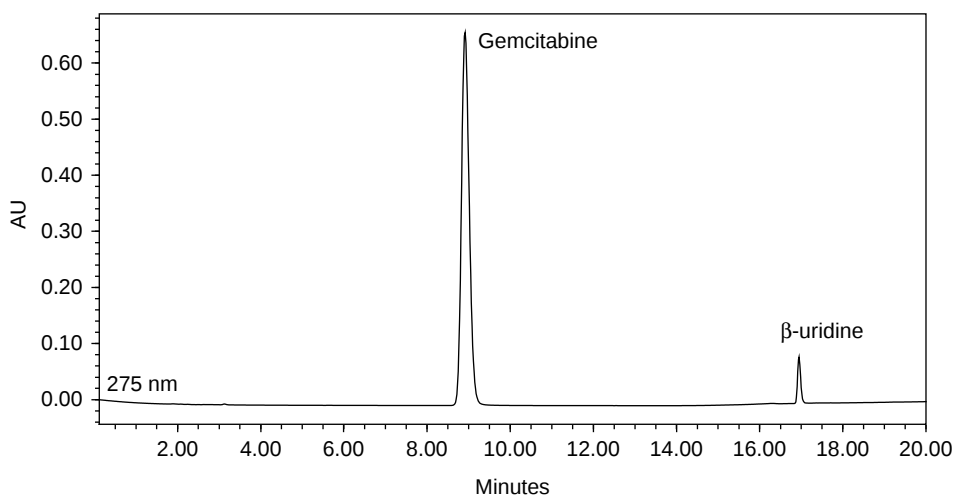


Figure 10 HPLC chromatogram obtained on a solution of gemcitabine hydrochloride in pH 3.2 acetate buffer stressed at 70°C for 2 days.

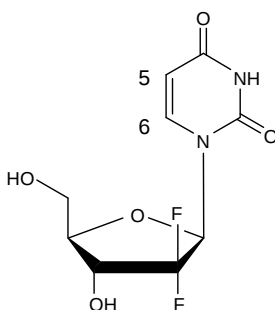


Figure 11 Structure of the β -uridine analog of gemcitabine.

therefore changed to 205 nm and the stressed sample was re-analyzed; the chromatogram is shown in Figure 12. At 205 nm, two additional impurities were detected. While isolating the two impurities for spectroscopic characterization (using a preparative HPLC column with a different mobile phase and gradient), a third impurity was discovered. This third impurity was found to co-elute with the parent gemcitabine peak on the analytical HPLC method. Isolation, structural characterization, and degradation studies of the three additional impurities confirmed that they were intermediates in the formation of the β -uridine analog. The structures and proposed mechanism are given in Scheme 1.

Structural information of stress-induced degradation products can also be used to assess the potential for formation of toxic/genotoxic degradation products. Both ICH guidelines on impurities (Q3A and Q3B) specifically address the issue of potential toxic impurities (114,115):

However, analytical procedures should be developed for those potential impurities that are expected to be unusually potent, producing toxic or pharmacological effects at a level not more than the identification threshold.

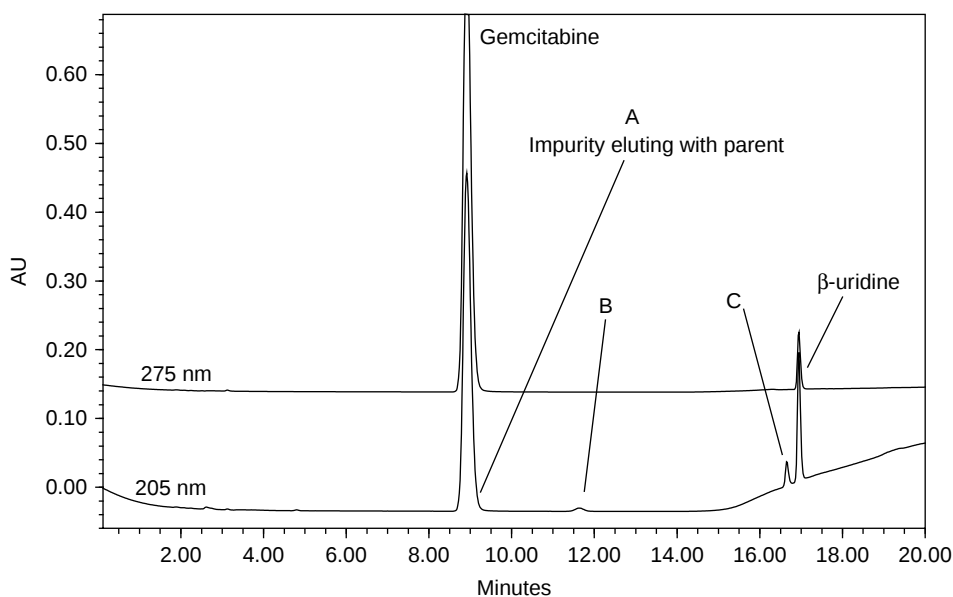
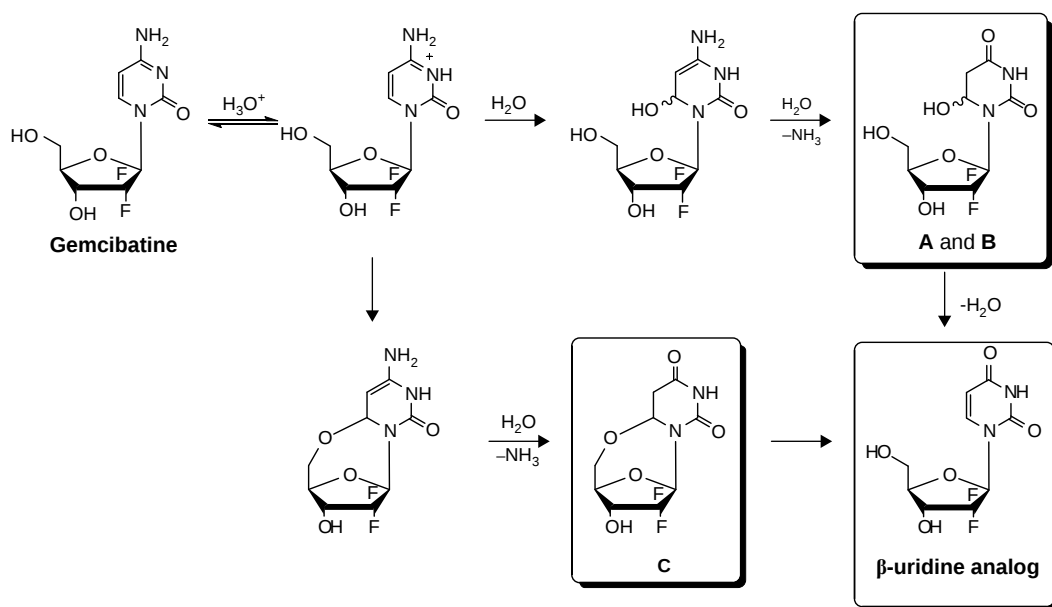


Figure 12 HPLC chromatograms obtained on a gemcitabine hydrochloride in pH 3.2 acetate buffer stressed at 70°C for 2 days.



Scheme 1 Proposed deamination mechanism of gemcitabine in the acidic aqueous solution.

Determining the structures of the major degradation products can reveal whether or not a known carcinogen or toxic compound is or might possibly be formed. When the structure(s) are novel, however, one cannot know for certain whether or not the compound will be unusually potent or toxic from the structure alone. Nonetheless, certain functional groups are often

regarded as potentially [geno]toxic (119,120), and scientists in the field of toxicology routinely evaluate compounds for toxicological potential on the basis of structure; software programs have been specifically developed for this purpose [e.g., TOPKAT (121), MultiCASE (122), DEREK (123)]. For a more thorough discussion on this topic, see chapter 19 and (145).

Determining structures of degradation products formed during stress testing can also be useful for preclinical discovery efforts during structure–activity relationship investigations (46,124). An understanding of the parts of the molecule that are labile or susceptible to degradation can help in the design of less reactive, more stable analogs. The development of a stable formulation is also aided by an understanding of the reactive parts of the drug molecule. Drug–excipient compatibility studies (which are a form of stress testing) often lead to new, unknown degradation products. The rational development of a stable formulation is greatly aided by a chemical understanding of the reactions leading to degradation. See chapter 11 for a thorough discussion of drug–excipient compatibility studies.

Elucidation of the structures of degradation products is typically a collaborative research undertaking that involves analytical, organic, and physical chemistry knowledge combined with spectroscopic information. It is not possible to define a precise process for the determination of an unknown degradation product, but common approaches are apparent in the modern laboratory as evidenced by the literature. Once a degradation product has been targeted for structure elucidation, a decision is made as to whether or not to begin spectroscopic characterization of the unknown in the mixture or after isolation and purification. Although the UV spectrum of the unknown can be obtained via HPLC with photodiode array UV detection, the first piece of spectroscopic information that is often sought is the molecular weight. HPLC/MS (especially with electrospray or atmospheric pressure chemical ionization in either the negative or the positive ion mode) is commonly used in the pharmaceutical industry to obtain molecular weight information. Often times, the MS information (i.e., molecular weight and fragmentation information) can provide enough information to allow proposal of a likely structure (125); at times, the structure can be confidently proposed based on this information along with a supporting chemical rationale (e.g., expected degradation chemistry based on the structure). Accurate mass MS can be especially useful in that it not only provides the molecular weight, but can also provide the molecular formula (126). Knowledge of organic chemistry and the conditions that led to the formation of the unknown are critical to making plausible structural proposals. When a structure is proposed, often times the proposal can be tested by comparison to “authentic standards” (i.e., if the compound has already been prepared via efforts of the synthetic organic chemists or if such a compound can easily be synthetically prepared). If the chromatographic retention and MS information (or, alternatively, the UV spectrum) of the unknown match the authentic standard, it is usually regarded as sufficient evidence to establish the structure. Alternatively, when the structure proposal involves a novel structure that is not available or readily prepared synthetically, further characterization is needed and consideration is given to isolation and purification. HPLC/NMR, while expensive and technologically demanding, is maturing as a technique (13) and is now used in some laboratories as an alternative to isolation and purification (127,128). Nonetheless, HPLC/NMR is still generally used for situations where the unknowns are difficult to isolate and purify or when samples amounts are limited (129,130).

Isolation and purification of unknowns can be accomplished by a variety of techniques (e.g., preparative HPLC or TLC, flash chromatography, extraction, etc.) (131,132). Preparative HPLC, RP or NP, is probably the most widely used technique in the pharmaceutical industry for purification of milligram to gram quantities of low-level impurities, although the use of preparative supercritical fluid chromatography (SFC) is growing, especially due to use of a carbon dioxide mobile phase that offers environmental and cost benefits compared with traditional liquid chromatography solvents (133,134). Once an unknown is isolated, spectroscopic characterization by MS and NMR is usually sufficient to unambiguously assign structures. UV, IR, and/or Raman are often used to identify specific chromophores or functional groups. Spectroscopic characterization of unknown impurities leading to structure elucidation is a process that has been discussed extensively elsewhere (135–140) and need not be reproduced here.

In summary, we advocate a chemistry-guided approach to developing an understanding of the intrinsic stability characteristics of a pharmaceutical compound. Acquiring structural information of the major degradation products observed during stress-testing facilitates such an approach.

INTRINSIC STABILITY: PATHWAYS OF DEGRADATION

As defined by ICH and by the earlier discussion, “stress testing is useful to help identify the likely degradation products, which can in turn help establish the degradation products and pathways and the intrinsic stability of the molecule and validate the stability indicating power of the analytical procedures used.” Establishing the pathways of degradation is critical, therefore, to developing an understanding of the intrinsic stability of the molecule, and degradation pathway information provides a scientific foundation for the validation of the stability indicating power of the analytical methodology.

The determination of degradation product structures (discussed previously in the chapter) provides the critical information needed to allow proposal (and testing) of plausible degradation pathways. The importance of this approach is seen in the example of LY297802 (discussed previously in this chapter in the section “Solution: Oxidative Stress-Testing Results”). The example of LY297802 shows the importance of determining degradation product structures in order to understand the degradation pathways.

Another example can be found in the case of duloxetine hydrochloride. Duloxetine hydrochloride is a compound that is unstable under acidic conditions (141), degrading to four main compounds (142). The structures of the major degradation products were determined and are shown in Figure 13. The structures revealed that the aryl ether linkage of duloxetine is acid labile, from which degradation pathways were proposed. The structures and proposed pathways do not implicate other nonobserved degradation products, as was the case for both LY297802 and gemcitabine hydrochloride (see section “Summary”). Thus, the structures and pathways help to provide assurance that the major degradation products are being resolved and detected. The critical step in the degradation pathways of duloxetine is the formation of a cationic intermediate (Fig. 14). The proposal of the cationic intermediate is important for a few reasons. First, it shows that there is one primary acid-labile site in the molecule. Second, it allows for an understanding of the instability; that is, it becomes apparent that the stability of

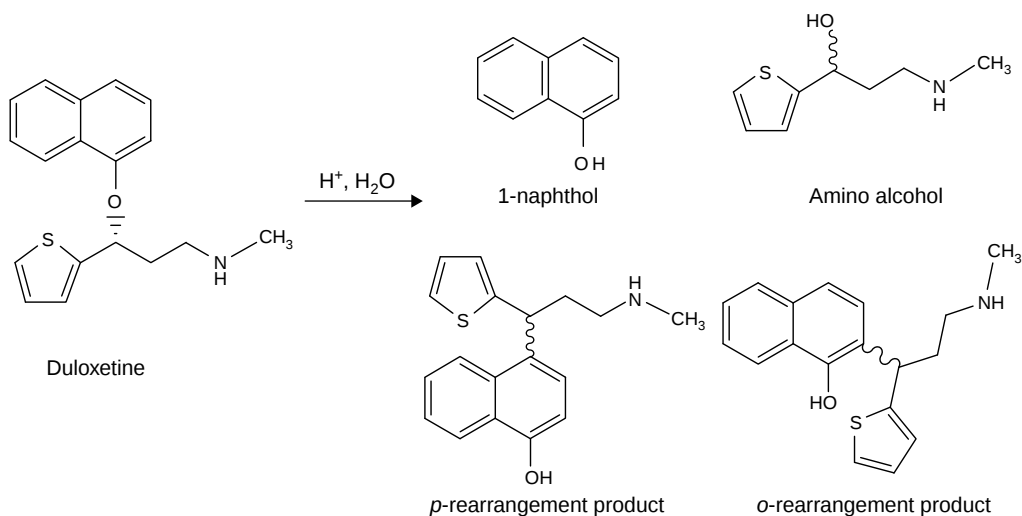


Figure 13 The structures of duloxetine and the four main acidic hydrolysis products.

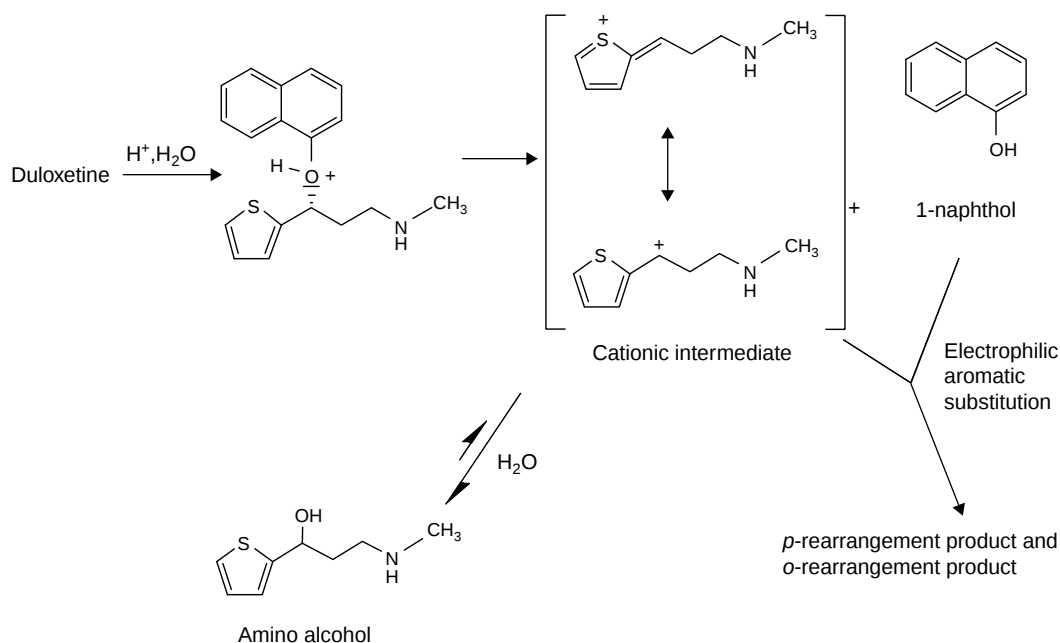


Figure 14 Proposed acid-catalyzed degradation pathways for duloxetine.

the cationic intermediate is likely a major contributor to the acid instability of duloxetine. Such information can be important in designing ways to stabilize the compound, for example, liquid formulations using solvents with a low dielectric constant or solid formulations with excipients and packaging that minimize the levels of moisture or incorporate basic excipients. Degradation pathway information can also be useful for new drug discovery efforts involving modifications to the chemical structure in order to reduce the ability of the adjacent aromatic group (i.e., the thiophene) to delocalize and stabilize the cation. The ability of thiophene to stabilize the charge is due, in large part, to the location of sulfur atom in relation to the site of attachment of the alkyl side chain.

Stress-testing studies should also be conducted on the formulated drug product because drugs can, and often do, degrade differently in the presence of excipients. One such example is duloxetine hydrochloride, the molecule discussed in the preceding paragraph. Because duloxetine is unstable in solution at pH values <2.5 , enteric polymer-coated formulations were developed to prevent its acid degradation in the stomach and to provide for subsequent rapid disintegration and release in the small intestine (141). A tablet formulation coated with the enteric polymer hydroxypropyl methyl-cellulose phthalate (HPMCP) was originally developed, but a formulation consisting of pellets coated with the enteric polymer hydroxypropyl methylcellulose acetate succinate (HPMCAS) contained in a capsule was the desired market formulation. The structures of the enteric polymers are given in Fig. 15.

During stress testing (60°C for 14 days) of the HPMCAS-coated pellet formulation, an unknown impurity eluting after duloxetine was detected by HPLC. This impurity was also detected at significant levels in stability samples stored at either 30°C/60% RH or at 40°C/75% RH. Subsequent analysis of stability samples of HPMCP-coated tablets indicated the presence of a different impurity that also eluted after duloxetine. Structural characterization of these impurities indicated that the impurity formed in the HPMCAS-coated pellets was a duloxetine succinamide and that the impurity formed in the HPMCP-coated tablets was a duloxetine phthalamide (see Fig. 16 for structures). These impurities are the result of an interaction of duloxetine with the enteric polymers. The formation of the duloxetine succinamide in the

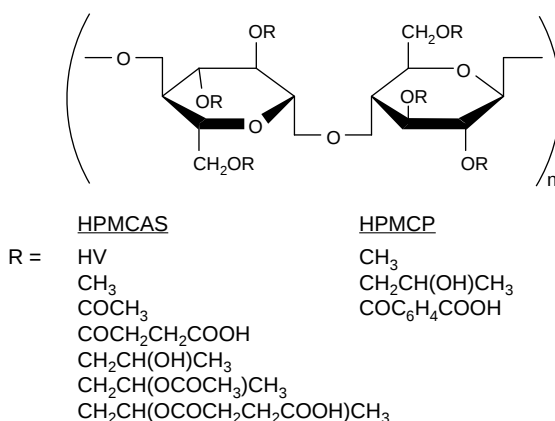


Figure 15 Structures of enteric polymers HPMCAS and HPMCP.

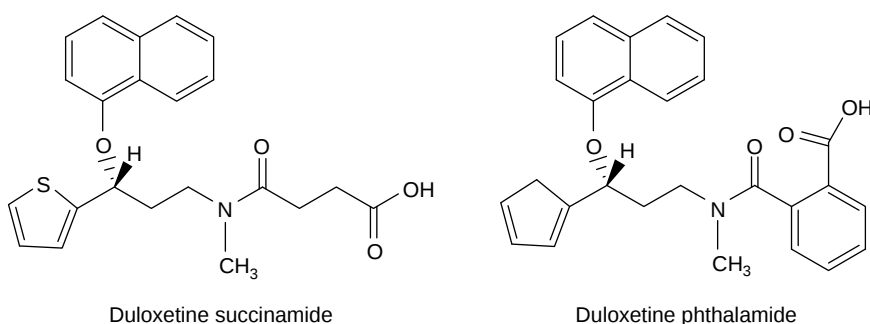


Figure 16 Structures of duloxetine succinamide and duloxetine phthalamide, the major impurities resulting from interaction between duloxetine and the enteric coatings HPMCAS and HPMCP during stress testing or long-term stability studies.

commercial formulation was minimized by increasing the thickness of the barrier layer that separates duloxetine from the enteric coating. In this case, early stress testing of the formulation uncovered a significant degradation problem that was easily corrected by making a relatively minor change in the formulation. Without knowledge of the *structure* of the impurity and its origin, it would have been difficult to minimize its formation.

INTERPRETATION OF THE RESULTS OF STRESS TESTING

One of the more interesting and challenging aspects of stress testing involves interpreting the results of stress testing such that the data becomes meaningful to the development process. For example, what levels of instability are indicative of problems for analytical handling, manufacturing/processing, formulation, patient “in use,” and storage and distribution? Can stress-testing results be easily interpreted to conclude whether or not a compound will have high-, medium-, or low-stability concerns for the development process? These questions are difficult as there are no (as yet) scientifically derived criteria for predicting the “developability” of a new drug entity. Such interpretive criteria would be very useful for the pharmaceutical development process.

Attempts have been made to use stress-testing results to classify compounds as extremely labile, very labile, labile, and stable (143). The basis for this classification system was the personal experience of the authors. In this section, we will attempt to outline and discuss some of

the factors that need to be considered when attempting to interpret the results of stress testing, and to provide references to other resources. It is our opinion that insufficient information has been gathered to provide a definitive classification system.

Interpretation of stress-testing results has several different facets. An obvious consideration is the degradation rate under a particular condition. The rates of degradation are critically important for determination of shelf life (although stress-testing results are, in general, not accurate enough to be used for shelf-life determination) and for handling considerations during manufacturing, formulation, and analysis. In addition to the kinetic interpretation, it is important to understand what the stress-testing results indicate about mechanism of degradation (e.g., from the structures of degradation products and the degradation pathways involved). Such information is important in designing the appropriate degradation-control strategies (12) (e.g., developing a stable formulation, appropriate packaging and storage conditions, and relevant analytical methodologies).

Solid State

There are three main stress conditions evaluated during stress testing in the solid state: temperature, humidity, and photostability. The stress of elevated temperature is perhaps the stress condition that has historically been considered to lend itself most directly to predictive interpretation. This assumes, of course, that Arrhenius kinetics are observed within the range of long-term storage temperature to the stressed temperature(s). Assuming Arrhenius kinetics and reasonable energies of activation, predictions can be made to relate the amount of degradation and increases of individual degradation products at the stress temperature to the long-term storage temperature. As discussed previously in the chapter (see section "Thermolytic Degradation"), the consideration of relative humidity is essential to enable the Arrhenius-derived predictions of degradation rates at different temperatures. Thus, it is possible to build accurate models of degradation rates using stress testing and a modified Arrhenius equation that incorporates relative humidity (2).

The solid-state stress of exposure to light (i.e., ultraviolet and visible radiation) is guided by the ICH guideline on photostability (101). The ICH guideline is primarily directed toward confirmatory photostability testing, which outlines photoexposure levels for the purpose of identifying potential photostability problems that may be encountered during storage and distribution of the marketed product. Most of these types of problems can be addressed through modifications of packaging, labeling, and/or formulation (with some associated expense). Very little guidance is given to the interpretation of photostress studies (which the guideline refers to as "forced degradation testing") in relation to the development process (101):

The forced degradation studies should be designed to provide suitable information to develop and validate test methods for the confirmatory studies. These test methods should be capable of resolving and detecting photolytic degradants that appear during the confirmatory studies. When evaluating the results of these studies, it is important to recognize that they form part of the stress testing and are therefore not designed to establish qualitative or quantitative limits for change.

Thus, photostressing studies are primarily useful for developing an understanding of the photochemistry of the drug and for developing appropriate analytical methodologies. See chapters 7 and 8 for further discussion of this topic.

One possible use of photostability/photostressing studies is the assessment of the potential for problems in manufacturing and analytical handling. It has been recommended by EFPIA that 100,000 lux-hr is a reasonable amount of light exposure to determine whether or not special precautions should be considered during manufacturing (100). This level of light exposure should also provide a reasonable estimation of problems that might be encountered during analytical and formulation development. Of course, the potential for exposure to UV light may

also need to be assessed depending on the lighting conditions of the analytical laboratories and the manufacturing facility. The best way to assess the potential light exposure would be to make spectroradiometric measurements of both the UVA and visible regions in the actual laboratories and manufacturing facilities.

Solution: Acid/Base Stress-Testing Results

These results will indicate whether or not the drug molecule has a particular instability in aqueous conditions as a function of pH. An approximate pH-rate profile can sometimes be constructed from the data, but caution should be exercised because it is not uncommon for a drug compound to be relatively insoluble in some pH ranges, and some samples may require co-solvents in order to achieve dissolution. If precise information on either kinetics or the precise pH range of maximum stability is desired (e.g., for a compound that will have a liquid formulation), further studies in which the solvents, the ionic strength, the buffer type, and the concentration are carefully controlled, should be conducted.

Stress-testing information can be relevant for ADME concerns. Critical evaluation of the pH 1–2 degradation rate data can help assess the potential need for enteric coating of drugs to be administered orally. The pH of gastric fluid (worst case) can be roughly simulated using 0.1 N HCl (pH 1). While transit times in the stomach are highly variable (dependent on variables such as % liquids/solids ingested, amount ingested, fasting level), most literature values fall within the time of 30 minutes to 4 hours (144). Assuming a worst case transit time (4-hour exposure to the acidic environment in the stomach), it seems reasonable to consider the potential need for an enteric-coated formulation if a loss of potency of ~10–20% or greater is observed after 4 hours at 37–40°C in 0.1 N HCl; assuming first-order kinetics of degradation, this degradation rate would correspond to a half-life of ~26 hours. Consideration of the potential need for enteric coating would involve many factors. For example, do the acidic degradation products raise questions of safety or efficacy? How rapid is the absorption? What is the half-life of the compound in the body? What are the pharmacological needs for efficacy of the parent? These questions need to be addressed on a case-by-case basis in an interdisciplinary manner (e.g., scientists from ADME, pharmacokinetics, analytical, formulations, and medical/clinical areas).

Evaluation of solution stability under neutral to moderately basic (e.g., pH 6–9) can help in assessing the potential for nonenzymatic breakdown of the compound under physiologically relevant conditions (e.g., pH 7.5). Such information can be useful not only for metabolism studies, but also for pharmacokinetic concerns (i.e., understanding how long a compound might remain intact *in vivo*) and what degradation products might form under such conditions. Thus, for example, if a compound degrades at pH 7.5 at a rate significantly faster than the *in vivo* half-life of the compound, the nonenzymatically formed degradation products will likely contribute to the metabolic profile of the compound.

Evaluation of degradation rates under different pH conditions also provides needed information related to analytical concerns. For example, how much degradation might occur during the analytical work-up under specific pH conditions? Can samples be prepared and held at room temperature for a specified length of time without appreciable degradation? Do samples need to be refrigerated in order to maintain stability during analysis? Answers to these questions are fairly straightforward if the analytical constraints are defined.

Solution: Oxidative Stress-Testing Results

It can be difficult to translate oxidative stress-testing results into accurate predictions of the susceptibility of a compound to oxidation. This is partially because oxidative mechanisms can be quite diverse and complex and oxidative degradation may not follow typical Arrhenius kinetic models, especially in solution. For a more in-depth discussion of this subject, see chapter 6 and the section “Hydrolytic Degradation.”

In chapter 6, Harmon suggests that the two most relevant tests for prediction of oxidative susceptibility of a drug compound involves the use of radical initiators (such as AIBN) for testing susceptibility to autoxidation and the use of dilute hydrogen peroxide for testing susceptibility to oxidation by peroxides (e.g., from excipients). Boccardi has asserted that, assuming a test involving the use of AIBN in an equimolar basis with the drug, after 48 hours at 40°C, compounds that are sensitive to autoxidation will likely be degraded >10% whereas compounds that are relatively stable to autoxidation will likely be degraded no more than a few percent (91). A scientifically sound approach to a more accurate quantitative assessment of the oxidative susceptibility of pharmaceuticals would be a valuable contribution to the future of pharmaceutical development.

In the case of stress testing using hydrogen peroxide, it is difficult to associate a percent degradation with a classification of oxidizability. If there are amines present in the molecule, especially tertiary amines, oxidation is usually rapid if the amine is uncharged (e.g., the free base). A protonated cationic amine is protected and the oxidation rate will be greatly reduced. In general, however, it is the experience of the authors that if a 0.3% solution of hydrogen peroxide in the presence of the drug in an unbuffered water solution induces <5% degradation in 24 hours at room temperature, the compound is not particularly sensitive to peroxides and will likely not require special considerations for development. Alternatively, ~20% or more degradation in 24 hours may indicate a particularly sensitive compound that could require special efforts in the formulation and/or storage conditions to ensure oxidative stability.

SUMMARY

Stress testing is an important tool for the prediction of stability-related problems. The results of stress testing can be useful to many areas of pharmaceutical research and development beyond the obvious areas of analytical, formulation, and packaging development. Well-designed stress-testing studies can lead to a thorough understanding of the *intrinsic stability characteristics* of the drug molecule, allowing a QbD-type approach where thorough knowledge and understanding are the foundation for building the stability control strategy. We advocate a chemistry-guided approach to stress testing, where structures of the major degradation products are determined and degradation pathways are proposed and tested. The quantitative interpretation of stress-testing results is an underdeveloped area that has made significant progress in the last six years (since the publication of the first edition of this book); continuing research is expected to contribute to new understanding and better tools for the future.

REFERENCES

1. Waterman KC, Adami RC. Accelerated aging: prediction of chemical stability of pharmaceuticals. *Int J Pharm* 2005; 293: 101–25.
2. Waterman KC, Carella AJ, Gumkowski MJ, et al. Improved protocol and data analysis for accelerated shelf-life estimation of solid dosage forms. *Pharm Res* 2007; 24: 780–90.
3. Waterman KC. Understanding and predicting pharmaceutical product shelf-life. In: Huyn-Ba K, ed. *Handbook of Stability Testing in Pharmaceutical Development: Regulations, Methodologies, and Best Practices*. New York, NY: Springer, 2009.
4. Kumar L, Amin A, Bansal AK. Salt selection in drug development. *Pharm Tech* 2008; 128–46.
5. Chadwick EW, Tran, CT, Roy R, et al. Designing green oxidation catalysts for purifying environmental waters. *J Am Chem Soc* 2010; 132: 9774–81.
6. Ryabov AD, Collins TJ. Mechanistic considerations on the reactivity of green Fe(III)-TAML activators of peroxides. *Adv Inorg Chem* 2009; 61: 471–521.
7. International Conference on Harmonization Q1A(R2): Stability Testing of New Drug Substances and Products (Second Revision), 2003.
8. International Conference on Harmonization, Guidance for Industry: Pharmaceutical Development, Q8, May 2006.
9. International Conference on Harmonization, Guidance for Industry: Quality Risk Management, Q9, November 2005.

10. International Conference on Harmonization, Guidance for Industry: Pharmaceutical Quality System, Q10, April 2009.
11. Baertschi, SW. Forced degradation and its relation to real time drug product stability. In: Huyn-ba K, ed. *Pharmaceutical Stability Testing to Support Global Markets*. New York, NY: Springer, 2009.
12. Olsen BA, Baertschi SW. Strategies for investigation and control of process-related and degradation-related impurities in pharmaceuticals. In: Ahuja S, Alsante KM, eds. *Handbook of Isolation and Characterization of Impurities in Pharmaceuticals*. Volume 5 of Separation Science and Technology. San Diego, CA: Academic Press, 2003.
13. Baertschi SW. Analytical methodologies for discovering and profiling degradation-related impurities. *Trends Anal Chem* 2006; 25: 758–67.
14. Huyn-ba K, ed. *Handbook of Stability Testing in Pharmaceutical Development*. New York: Springer, 2009.
15. Alsante KM, Martin L, Baertschi SW. A stress testing benchmarking study. *Pharma Technol* 2003; 27: 60–72.
16. Olsen BA, Castle BC, Myers DP. Advances in HPLC technology for the determination of drug impurities trends. *Anal Chem* 2006; 25: 796–805.
- 17a. Snyder LR, Glajch JL, Kirkland JJ. *Practical HPLC Method Development*. New York: John Wiley and Sons, 1988.
- 17b. Snyder LR, Glajch JL, Kirkland JJ. *Introduction to Modern Liquid Chromatography*, 3rd edn, New York: John Wiley and Sons, 2010.
18. Alsante KM, Ando A, Brown R, et al. The role of degradant profiling in active pharmaceutical ingredients and drug products. *Adv Drug Deliv Rev* 2007; 59: 29–37.
19. Monkhouse DC, Maderich A. Whither compatibility testing? *Drug Dev Ind Pharm* 1989; 15: 2115–30.
20. Serajuddin ATM, Thakur AB, Ghoshal RN, et al. Selection of solid dosage form composition through drug–excipient compatibility testing. *J Pharm Sci* 1999; 88: 696–704.
21. Crowley P, Martini L. Drug–excipient interactions. *Pharm Technol Europe* 2001; 13: 26–8, 30–2, 34.
22. Akers MJ. Excipient–drug interactions in parenteral formulations. *J Pharm Sci* 2002; 91: 2293–300.
23. Selzer T, Radau M, Kreuter J. The use of isothermal heat conduction microcalorimetry to evaluate drug stability in tablets. *Int J Pharm* 1999; 184: 199–206.
24. Wissing S, Craig DQM, Barker SA, Moore WD. An investigation into the use of stepwise isothermal high sensitivity DSC as a means of detecting drug–excipient incompatibility. *Int J Pharm* 2000; 199: 141–50.
25. Schmitt EA, Peck K, Yang S, Geoffroy J-M. Rapid, practical and predictive excipient compatibility screening using isothermal microcalorimetry. *Thermochim Acta* 2001; 380: 175–83.
26. McDaid FM, Barker SA, Fitzpatrick S, Petts CR, Craig DQM. Further investigations into the use of high sensitivity differential scanning calorimetry as a means of predicting drug–excipient interactions. *Int J Pharm* 2003; 252: 235–40.
27. Reynolds DW, Facchine KL, Mullaney JF, et al. Available guidance and best practices for conducting forced degradation studies. *Pharm Technol* 2002; 26: 48–54.
28. International Conference on Harmonization, Guidance for Industry: Pharmaceutical Development, Q8, Annex, November 2006.
29. Baertschi SW. Analytical methodologies for discovering and profiling degradation-related impurities. *TrAC Trends Anal Chem* 2006; 25: 758–67.
30. Jansen PJ, Smith KW, Baertschi SW. Stress testing: analytical considerations. In: Baertschi SW, ed. *Pharmaceutical Stress Testing: Predicting Drug Degradation*. Vol. 153. New York: Taylor & Francis, 2005: 141–71.
31. Baertschi SW, Nussbaum MA, Jansen PJ. Role of “mass balance” in pharmaceutical stress testing. In: Baertschi SW, ed. *Pharmaceutical Stress Testing: Predicting Drug Degradation*. Vol. 153. New York: Informa Healthcare, 2005: 181–204.
32. Waterman KC, Adami RC, Hong J. Impurities in drug products. In: Ahuja, S, Alsante, KM, eds. *Handbook of Isolation and Characterization of Impurities in Pharmaceuticals*. New York: Academic Press, 2003: 75–88.
33. Zannou EA, Ji Q, Joshi YM, Serajuddin ATM. Stabilization of the maleate salt of a basic drug by adjustment of microenvironmental pH in solid dosage form. *Int J Pharma* 2007; 337: 210–18.
34. Guerrieri P, Salameh A, Taylor L. Effect of small levels of impurities on the water vapor sorption behavior of ranitidine HCl. *Pharma Res* 2007; 24: 147–56.
35. Salameh AK, Taylor LS. Deliquescence in binary mixtures. *Pharma Res* 2005; 22: 318–24.

36. Dieter K. In: Grimm W, Thoma K, Krummen K, eds. *Stability Testing in the EC, Japan, and the USA: Scientific and Regulatory Requirements*. Chapter 4. Stuttgart: Wissenschaftliche Verlagsgesellschaft mbH, 1993.
37. Connors KA, Amidon GL, Stella VL. *Chemical Stability of Pharmaceuticals: A Handbook for Pharmacists*, 2nd edn. New York: Wiley, 1986: 19; reference 3.
38. Waterman KC. Accelerated Stability Assessment Program (ASAP): using science to set shelf-life. Presented at the AAPS workshop on Stability Testing in Pharmaceutical Development, AAPS Annual Meeting/FIP World Congress. November 14, 2010: New Orleans, LA.
39. Jerussi RA. Stability testing and generics. *J cGMP Compliance* 1999; 3: 28–32.
40. Connors KA. *Chemical Kinetics: The Study of Reaction Rates in Solution*. New York: VCH Publishers, 1990: 191; Table 5–1.
41. Kennon LJ. Use of models in determining chemical pharmaceutical stability. *J Pharm Sci* 1964; 53(7): 815–18.
42. USP <1150> PHARMACEUTICAL STABILITY. USP 32—National Formulary 27 S2, 2010.
43. Davis JS. The dating game. Proprietary Association's 12th Manufacturing Controls Seminar, New Jersey, October 5–6, 1978.
44. Davis JS. Criteria for accelerated stability testing. FDA/ASQC Seminar, Chicago, IL, March 11, 1991.
45. Yang W-H, Roy SB. Projection of tentative expiry date from one-point accelerated stability testing. *Drug Dev Ind Pharm* 1980; 6: 591–604.
46. MacFaul PA, Ruston L, Wood JM. Activation energies for the decomposition of pharmaceuticals and their application to predicting hydrolytic stability in drug discovery. *Med Chem Commun* 2011; 2: 140–2.
47. Unpublished data, personal communication from Hofer JD, Wolfe C., Eli Lilly and Company.
48. Waterman KC. Accelerated Stability Assessment Program (ASAP): Using Science to Set Shelf Life. Presented at the AAPS Workshop on Stability Testing in Pharmaceutical Development. November 14, 2010. New Orleans, LA.
49. The assumption that 6 months at 40°C/75% RH translates to 2 years at 25°C/60%RH assumes an E_a of 17 kcal/mol.
50. Connors KA, Amidon GL, Stella VJ. Solid-state chemical decomposition. In: *Chemical Stability of Pharmaceuticals: A Handbook for Pharmacists*, 2nd edn. Chapter 6. New York: John Wiley and Sons, 1986: 116–19.
51. Monkhouse DC, Campen LV. Solid-state reactions—theoretical and experimental aspects. *Drug Dev Ind Pharm* 1984; 10: 1175–276.
52. Guerrieri P, Salameh A, Taylor L. Effect of small levels of impurities on the water vapor sorption behavior of ranitidine HCl. *Pharma Res* 2007; 24: 147–56.
53. Salameh AK, Taylor LS. Deliquescence in binary mixtures. *Pharma Res* 2005; 22: 318–24.
54. Guierrieri P, Taylor LS. Role of salt and excipient properties on disproportionation in the solid-state. *Pharma Res* 2009; 26: 2015–26.
55. Grimm VW. Stabilitätsprüfung pharmazeutischer Zubereitungen. *Pharma Ind* 1975; 37: 815–25.
56. Witthaus G. Accelerated storage tests: predictive value. In: Breimer DD, Speiser P, eds. *Topics in Pharmaceutical Sciences*. Amsterdam, The Netherlands: Elsevier, 1981: 275–90.
57. Sims JL, Carreira JA, Carrier DJ, et al.. A new approach to accelerated drug–excipient compatibility testing. *Pharma Dev Technol* 2003; 8: 119–26.
58. Baertschi SW, Dorman DE, Occolowitz JL, et al. Isolation and characterization of degradation products arising from aqueous degradation of cefaclor. *J Pharm Sci* 1997; 86: 526–39.
59. Dorman DE, Lorenz LJ, Occolowitz JL, et al. Isolation and structure elucidation of the major degradation products of cefaclor in the solid state. *J Pharm Sci* 1997; 86: 540–9.
60. Olsen BA, Perry FM, Snorek SV, Lewellen PL. Accelerated conditions for stability assessment of bulk and formulated cefaclor monohydrate. *Pharm Dev Technol* 1997; 2: 303–12.
61. Baertschi SW, Elder D, Kleinman M, et al. Strategies for addressing potentially genotoxic degradants in active pharmaceutical ingredients and formulated products: a PhRMA white paper. Draft guidance, in preparation, 2010.
62. Greenspan, I. Humidity fixed points of binary saturated aqueous solutions. *J Res National Bureau Standards A. Phys Chem*, 1977; 81A: 89–96.
63. Stewart PJ, Tucker IG. Prediction of drug stability. Part 2. Hydrolysis *Aust J Hosp Pharm* 1985; 15: 11–16.
64. Waterman KC, Adami RC, Antipas AS, et al. Hydrolysis in pharmaceutical formulations. *Pharm Dev Tech* 2002; 7: 113–46.

65. Mabey W, Mill T. Hydrolysis of organic compounds. In: Hutzinger O, ed. *The Handbook of Environmental Chemistry Volume 2/Part D*. Berlin: Springer-Verlag.
66. Mabey W, Mill T. Critical review of the hydrolysis of organic compounds in water under environmental conditions. *J Phys Chem Data* 1978; 7: 383–415.
67. Payne GB, Deming PH, Williams PH. Reactions of hydrogen peroxide. VII. Alkali-catalyzed epoxidation and oxidation using a nitrile as co-reactant. *J Org Chem* 1961; 26: 659–63.
68. Laus G. Kinetics of acetonitrile-assisted oxidation of tertiary amines by hydrogen peroxide. *J Chem Soc Perkins Trans* 2001; 2: 864–8.
69. Skibic MJ, King LA, Khan M, et al. Artifactual formylation of duloxetine hydrochloride by acetonitrile in the presence of titanium dioxide and ultrasonication: implications for HPLC method development. *J Pharm Biomed Anal* 2010; 53: 432–9.
70. Reynolds DW. Available regulatory guidance and best practices for conducting forced degradation studies. *Forced Degradation Studies: Best Practices for the Pharmaceutical Industry*. Institute for International Research, Princeton, NJ, February 24–25, 2004.
71. Stewart PJ, Tucker IG. Prediction of drug stability. Part 2. Hydrolysis. *Aust J Hosp Pharm* 1985; 15: 11–16.
72. Graham DJ. Relationship between solvatochromic solvent polarity and various thermodynamic and kinetic data in mixed solvent systems. *J Chem Soc Faraday Trans* 1990; 86: 287–91.
73. Alfassi ZB, Padmaja S, Neta P, Huie RE. Solvent effects on the rate of reaction of $\text{Cl}_2(\text{dot})^-$ and $\text{SO}_4(\text{dot})^-$ radicals with unsaturated alcohols. *Int J Chem Kinet* 1993; 25: 151–9.
74. Manege LC, Ueda T, Hojo M, Fujio M. Concentrated salt effects on the rates of solvolyses involving carbocations as reaction intermediates in acetone–water mixed solvents. *J Chem Soc Perkin Trans* 1998; 2: 1961–5.
75. Ito O, Watanabe H. Effect of preferential solvation on reactivity of a free radical in binary solvent systems. *J Chem Soc Faraday Trans* 1994; 90: 571–4.
76. LePree JM, Connors KA. Solvent effects on chemical processes. 11. Solvent effects on the kinetics of decarboxylative dechlorination of *N*-chloro amino acids in binary aqueous-organic solvents. *J Pharm Sci* 1996; 85: 560–6.
77. Skwierzynski RD, Connors KA. Solvent effects on chemical processes. 8. Demethylation kinetics of aspartame in binary aqueous-organic solvents. *J Pharm Sci* 1994; 82: 1690–6.
78. Calmon J-P, Canavy J-L. Solvent effects on the kinetics of alkaline hydrolysis of dimethylacetylacetone. Part 1. Influence of alcohol–water mixtures. *J Chem Soc Perkin II* 1972; 8: 706–10.
79. Bates RG. Medium effects and pH in nonaqueous solvents. In: Coetzee JF, ed. *Solute–Solvent Interactions*. New York: Marcel Dekker, 1969: 45–96.
80. Watarai H, Suzuki N. Keto-enol tautomerization rates of acetylacetone in mixed aqueous media. *J Inorg Nucl Chem* 1974; 36: 1815–29.
81. Castells CB, Rafols C, Roses M, Bosch E. Effect of temperature on pH measurements and acid–base equilibria in methanol–water mixtures. *J Chromatogr A* 2003; 1002: 41–53.
82. Canals I, Portal JA, Bosch E, Roses M. Retention of ionizable compounds on HPLC 4. Mobile-phase pH measurement in methanol/water. *Anal Chem* 2000; 72: 1802–9.
83. Espinosa S, Bosch E, Roses M. Retention of ionizable compounds in high-performance liquid chromatography. IX. Modeling retention in reversed-phase liquid chromatography as a function of pH and solvent composition with acetonitrile–water mobile phases. *J Chromatogr A* 2002; 947: 47–58.
84. Johnson DM, Gu LC. Autoxidation and antioxidants. In: Swarbrick J, Boylan JC, eds. *Encyclopedia of Pharmaceutical Technology*. Vol. 1. New York: Marcel Dekker, 1988: 425–9.
85. Munson E, Schoenich C. Oxidation reactions in the solid state: mechanisms and comparison to solution chemistry. *Oxidative Degradation and Stabilization Conference*. Institute for International Research, Princeton, NJ, July 20–21, 2004.
86. Boccardi G. Autoxidation of drugs: prediction of degradation impurities from results of reaction with radical chain initiators. *Il Farmaco* 1994; 49: 431–5.
87. Boccardi G. Stress testing: oxidative susceptibility testing. In: Baertschi SW, ed. *Pharmaceutical Stress Testing: Predicting Drug Degradation*. Chapter 7. Boca Raton, FL: Taylor and Francis, 2005: 214
88. Wasylaschuk WR, Harmon PA, Wagner G. Evaluation of hydroperoxides in common pharmaceutical excipients. *Ibid*. 2007; 96: 106–16.
89. Hovorka SW, Schöneich C. Oxidative degradation of Pharmaceuticals: theory, mechanisms, and inhibition. *J Pharm Sci* 2001; 90: 253–69.

90. Waterman KC, Adami RC, Alsante KM, et al. Stabilization of pharmaceuticals to oxidative degradation. *Pharm Dev Technol* 2002; 7: 1–32.
91. Boccardi G, Oxidative susceptibility testing. In: Baertschi S, ed. *Pharmaceutical Stress Testing: Predicting Drug Degradation*. New York: Taylor & Francis, 2005: 223–5.
92. Brustugun HH, Tonnesen WK, Kjonniksen P. Photosensitized degradation of losartan potassium in an extemporaneous suspension formulation. *PDA J Pharma Sci Technol* 2000; 54: 136–43.
93. Gardner I, Zahid N, MacCrimmon D, Uetrecht JP. A comparison of the oxidation of clozapine and olanzapine to reactive metabolites and the toxicity of these metabolites to human leukocytes. *Mol Pharmacol* 1998; 53: 991–8.
94. Sikora A, Adamus J, Marcinek A. Disproportionation of clozapine radical: a link between one-electron oxidation of clozapine and formation of its nitrenium cation. *Chem Res Toxicol* 2007; 20(8): 1093–8.
95. Miller BL, Kuczera K, Schöneich C. One-electron photooxidation of *N*-methionyl peptides. Mechanism of sulfoxide and azasulfonium diastereomer formation through reaction of sulfide radical cation complexes with oxygen or superoxide. *J Am Chem Soc* 1998; 120: 3345–56.
96. Beal JL, Foster SB, Ashby MT. Hypochlorous acid reacts with the *N*-terminal methionines of proteins to give dehydromethionine, a potential biomarker for neutrophil-induced oxidative stress. *Biochemistry* 2009; 48: 11142–8.
97. Peskin AV, Turner R, Magzal GJ, et al. Oxidation of methionine to dehydromethionine by reactive halogen species generated by neutrophils. *Biochemistry* 2009; 48: 10175–82.
98. Jupp P. Introduction to forced degradation studies. presented at the 2nd Annual Conference on Forced Degradation, Informa Life Sciences, Amsterdam, Netherlands, January 29–30, 2008.
99. Baertschi SW, Draper JR, Jansen PJ, Smith WK. Oxidative susceptibility testing: rapid, comprehensive, screening techniques. Presented at the Institute for International Research conference on Forced Degradation, Short Hills, NJ, 2006.
100. Reed RA, Harmon P, Manas D, et al. The role of excipients and package components in the photostability of liquid formulations. *J Pharm Sci Technol* 2003; 57: 351–68.
101. Stability testing: photostability testing of new drug substances and products. International Conference on Harmonisation, Q1B, Nov 1996.
102. Thatcher SR, Mansfield RK, Miller RB, Davis CW, Baertschi SW. Pharmaceutical photostability: a technical and practical interpretation of the ICH guideline and its application to pharmaceutical stability: Part I. *Pharm Technol* 2001; 25: 98–110.
103. Anderson NH. Photostability testing: design and interpretation of tests on drug substances and dosage forms. In: Tønnesen HH, ed. *Photostability of Drugs and Drug Formulations*. Great Britain: Taylor & Francis, 1996: 307.
104. Baertschi SW and Thatcher SR. Sample presentation for photostability studies: problems and solutions. In: Piechocki J, ed. *Pharmaceutical Photostability and Stabilization Technology*. New York: Taylor & Francis, 2006: 186–8.
105. Thatcher SR, Mansfield RK, Miller RB, Davis CW, Baertschi SW. Pharmaceutical photostability: a technical and practical interpretation of the ICH guideline and its application to pharmaceutical stability: part II. *Pharm Technol* 2001; 25: 50–62.
106. Tønnesen HH, ed. *The Photostability of Drugs and Drug formulations*. Great Britain: Taylor & Francis, 1996.
107. Tønnesen HH, ed. *The Photostability of Drugs and Drug formulations*, 2nd edn. Boca Raton, FL: CRC Press LLC, 2004.
108. Piechocki J, ed. *Pharmaceutical Photostability and Stabilization Technology*. Drugs and the Pharm Science v. 163. Informa Healthcare (2007).
109. Albini A, Fasani E, eds. *Drugs: Photochemistry and Photostability*. Cambridge: Royal Society of Chemistry, 1998.
110. Tønnesen HH. Photodecomposition of drugs. *Encyclopedia of Pharmaceutical Technology*. New York: Marcel Dekker 2002: 2197–203.
111. Connors KA. *Chemical Kinetics: The Study of Reaction Rates in Solution*. New York: VCH Publishers, 1990.
112. Carstensen JT. *Drug Stability: Principles and Practices*, 2nd edn. New York: Marcel Dekker, 1995.
113. Such an assumption is consistent with principles laid out in ICH Q3A(R2) and Q3B(R2).
114. Impurities in new drug substances. International Conference on Harmonisation, Q3A(R2). Revised guideline. 2006.

115. Impurities in new drug products. International Conference on Harmonisation, Q3B(R2) Revised guideline. 2006.
116. Jansen PJ, Akers MJ, Amos RM, et al. The degradation of the anti-tumor agent gemcitabine hydrochloride in an acidic aqueous solution at pH 3.2 and identification of degradation products. *J Pharm Sci* 2000; 89: 885–91.
117. Wechter WJ, Kelly RC. The mechanism of the deamination of cytidine. *Collect Czech Chem Commun* 1970; 35: 1991–2002.
118. Notari RE, Witiak DT, DeYoung JL, Lin AJ. Comparative kinetics of cytosine nucleosides. Influences of a 6-methyl substituent on degradation rates and pathways in aqueous buffers. *J Med Chem* 1972; 15: 1207–14.
119. Ashby J, Tennant RW. Definitive relationships among chemical structure, carcinogenicity, and mutagenicity for 301 chemicals tested by the U.S. NTP. *Mutation Res* 1991; 257: 229–306.
120. Ashby J, Paton D. The influence of chemical structure on the extent and sites of carcinogenesis for 522 rodent carcinogens and 55 different human carcinogen exposures. *Mutation Res* 1993; 286: 3–7.
121. <https://www.chem.ac.ru/chemistry/soft/TOPKAT.en.html>
122. <https://www.multicase.com/publications.html>
123. <https://www.lhasalimited.org/derek.nexus/>
124. Di L, Kerns EH. Stability challenges in drug discovery. *Chem Biodiversity* 2009; 6: 1875–86.
125. Wu Y. The use of liquid chromatography–mass spectrometry for the identification of drug degradation products in pharmaceutical formulations. *Biomed Chromatogr* 2000; 14: 384–96.
126. Winger BE, Kemp CAJ. Characterization of pharmaceutical compounds and related substances by using HPLC FTICR–MS and tandem mass spectrometry. *Am. Pharm. Rev.* 2001; 4: 55–63.
127. Wilson ID, Griffiths L, Lindon JD, Nicholson JK. HPLC/NMR and related hyphenated NMR methods. In: Görög S, ed. *Identification and Determination of Impurities in Drugs*. New York: Elsevier, 2000: 299–322.
128. Peng SX. Hyphenated HPLC–NMR and its applications in drug discovery. *Biomed Chromatogr* 2000; 14: 430–41.
129. Sharman GJ, Jones IC. Critical investigation of coupled liquid chromatography–NMR spectroscopy in pharmaceutical impurity identification. *Magn Reson Chem* 2003; 41: 448–54.
130. Cummings PG, Offen P, Olsen MA, Kennedy-Gabb S, Zuber G. LC/MS, LC/NMR, FTIR: an integrated approach to impurity identification in pharmaceutical development formulation. *Am Pharm Rev* 2003; 6: 88, 90, 92.
131. Gorman PM, Jiang H. Isolation methods I: thin-layer chromatography. In: Ahuja S, Alsante KM, eds. *Handbook of Isolation and Characterization of Impurities in Pharmaceuticals, Separation Science and Technology*, Vol 5. Chapter 9. New York: Academic, 2003.
132. Guinn M, Bates R, Hritzko B, et al. Isolation methods II: column chromatography. In: Ahuja S, Alsante KM, eds. *Handbook of Isolation and Characterization of Impurities in Pharmaceuticals, Separation Science and Technology*, Vol 5. Chapter 10. New York: Academic, 2003.
133. Zelesky T. Supercritical fluid chromatography (SFC) as an isolation tool for the identification of drug related impurities. *Am Pharm Rev.* 2008; 11: 56–60, 62.
134. Terfloth G. Preparative isolation of impurities. *Analysis of Drug Impurities.* 2007; 215–34.
135. Tollsten L. HPLC/MS for drug impurity identification. In: Görög S, ed. *Identification and Determination of Impurities in Drugs*. New York: Elsevier, 2000: 266–97.
136. Burinsky DJ, Wang F. Mass spectral characterization. In: Ahuja S, Alsante KM, eds. *Handbook of Isolation and Characterization of Impurities in Pharmaceuticals, Separation Science and Technology*, Vol 5. Chapter 11. New York: Academic Press, 2003.
137. Lohr LL, Jensen AJ, Sharp TR. NMR characterization of impurities. In: Ahuja S, Alsante KM, eds. *Handbook of Isolation and Characterization of Impurities in Pharmaceuticals, Separation Science and Technology*, Vol 5. Chapter 12. New York: Academic Press, 2003.
138. Feinberg TN. Hyphenated characterization techniques. In: Ahuja S, Alsante KM, eds. *Handbook of Isolation and Characterization of Impurities in Pharmaceuticals, Separation Science and Technology*, Vol 5. Chapter 13. New York: Academic Press, 2003.
139. Alsante KM, Hatajik TD, Lohr LL, Santafianos D, Sharp TR. Solving impurity/degradation problems: case studies. In: Ahuja S, Alsante KM, eds. *Handbook of Isolation and Characterization of Impurities in Pharmaceuticals, Separation Science and Technology*, Vol 5. Chapter 14. New York: Academic, 2003.
140. Görög S. New safe medicines faster: the role of analytical chemistry. *Trends Anal Chem* 2003; 22: 7–8.

141. Jansen PJ, Oren PL, Kemp CA, Maple SR, Baertschi SW. Characterization of impurities formed by interaction of duloxetine HCL with enteric polymers hydroxypropyl methylcellulose acetate succinate (HPMCAS) and hydroxypropyl methylcellulose phthalate (HPMCP). *J Pharm Sci* 1998; 87: 81–5.
142. Bopp RJ, Breau AP, Faulkinbury TJ, et al. The rearrangement of duloxetine under mineral acid conditions. 206th National American Chemical Society Meeting, San Diego, CA; March 13, 1993; ORGN 111.
143. Singh S, Bakshi M. Guidance on conduct of stress tests to determine inherent stability of drugs. *Pharm Technol Online* (April) 2000: 1–14.
144. Dressman JB. Comparison of Canine and human gastrointestinal physiology. *Pharm Res* 1986; 3: 123–31.
145. Hillebrecht A, Muster W, Brigo A, et al. Comparative evaluation of in silico systems for Ames Test mutagenicity prediction: scope and limitations. *Chem Res Toxicol* 2011. DOI: 10.1021/tx2000398.

3 Stress testing: The chemistry of drug degradation

Steven W. Baertschi, Karen M. Alsante, and Dinos Santafianos

INTRODUCTION

In this chapter, we will examine the major mechanisms of chemical decomposition of pharmaceuticals in the context of common functional groups. The major mechanisms of chemical decomposition of pharmaceuticals include hydrolysis/dehydration, oxidation, isomerization/epimerization, rearrangements, decarboxylation, dimerization/polymerization, and photolysis and transformation products involving reaction with excipients/salt forms. In order to develop an understanding of the chemistry of such reactions, a basic knowledge of organic chemistry is needed. Providing such a background is not the purpose of this book, and therefore the reader may find it useful to consult more advanced organic chemistry textbooks and literature references. A good general organic chemistry reference is *Advanced Organic Chemistry* by J. March (1). Stewart and Tucker have provided excellent references for consideration of drug degradation mechanisms (2). An in-depth discussion of the various mechanisms is beyond the scope of this chapter, but some discussion is warranted.

The mechanisms of hydrolysis/dehydration, isomerization/epimerization, decarboxylation, rearrangements, and some kinds of polymerization reactions can be generalized into a condition that has been called “thermolytic” (3); these reactions are generally sensitive to temperature and can be accelerated by elevating the temperature under various conditions in the solid state (low and high humidity). *Hydrolytic* reactions, can be accelerated both by exposure to elevated temperature as and by exposure to different pH values in a broad pH range. *Oxidative* degradation of pharmaceuticals is generally the result of autoxidation (see section “Benzyl Groups,” later in this chapter, for additional discussion of autoxidation chemistry, and chap. 6). The rate of autoxidation may not always be accelerated by temperature in a predictable manner, although Waterman has shown that many oxidative degradation reactions will follow Arrhenius kinetics in the solid state if the effects of relative humidity are built into the Arrhenius equation (see chap. 2 for further discussion) (4,5). Autoxidation is driven by the formation of radicals (via initiators such as transition metals, low levels of peroxides, or molecular oxygen). Many polymerization reactions are the result of radical-initiated processes. Many drug oxidation reactions are also the result of reaction with peroxides, which may be present at low levels in some excipients and which may form over the time as a result of autoxidation. *Photolytic* reactions are initiated by the absorption of photons from exposure to various sources of light (e.g., sunlight, metal halide lamps, fluorescent lamps, or other indoor lighting sources), and therefore these reactions can be induced by exposure to photolysis sources emitting in the 290–800 nm region. More detailed discussions of the design of stress testing studies to evaluate these major degradation pathways (i.e., thermolytic, hydrolytic, oxidative, and photolytic) can be found in other chapters in this book (chaps. 2, 4, 6–8).

Common Degradation Pathways

While many pathways of degradation are obvious from basic organic chemistry principles, it is not uncommon to find surprising degradation chemistry leading to unexpected degradation products and pathways. As the field of degradation chemistry matures, and degradation pathways of various drug scaffolds, ring systems, and functional group arrangements are investigated and documented in the literature, the predictability of degradation pathways will dramatically improve and patterns will emerge.

A major step in this direction has been taken with the development of an online searchable drug degradation database, PharmaD3 (<http://d3.cambridgesoft.com/>). This chemical structure searchable database was started by Alsante and Baertschi, in collaboration with

Cambridgesoft™ (maker of ChemDraw™, Cambridge, MA) in 2005. The intent of the database is to be populated with drug degradation examples published either in the scientific literature or presented at scientific conferences. The database allows structure and name-based searching of the parent drug or the degradation product, and the conditions of the degradation and publication reference are included in the database. The database also allows for searching by molecular weight (MW) change, that is, the difference between the MW of the parent and the degradants. Since working on this chapter, 365 drugs and more than 1200 unique degradation products have been entered into the database. As this compilation grows, the data should provide a useful tool for the field of degradation chemistry, enabling searches of specific drugs and molecular scaffolds as well as uncovering patterns of degradation of specific functional groups and of drugs in general. As an example of the power of such an exercise, a search was conducted to determine the most common/frequent degradation pathways as a function of changes in MW from the parent to the degradant. Thus, the database was searched (6) for all examples of degradants that show MW changes from the parent of -60 to $+60$ amu, in 1 amu increments (7). The results of this effort are captured in Figure 1, where the number of degradants is plotted versus MW change.

The plot shown in Figure 1 reveals that there are patterns in degradation pathways, with certain MW changes occurring more frequently than others. Many of the MW changes are not surprising, as indicated in Table 1. For example, changes in the MW of $+16$ and $+32$ amu occur frequently, corresponding to the addition of 1 and 2 oxygen atoms, respectively. Likewise, a change in the MW of $+18$ or -18 amu can readily be explained by the addition or loss of water. The most common MW change is 0 amu, with more than 60 examples found in the database (epimerization, rearrangements, etc.). Interestingly, however, there are many MW changes for which the degradation chemistry leading to such a change are not as obvious. The MW change of $+58$ has been observed in formulated products by at least three pharmaceutical companies (Eli Lilly, Pfizer, and GSK), who each independently researched the problem and found that the

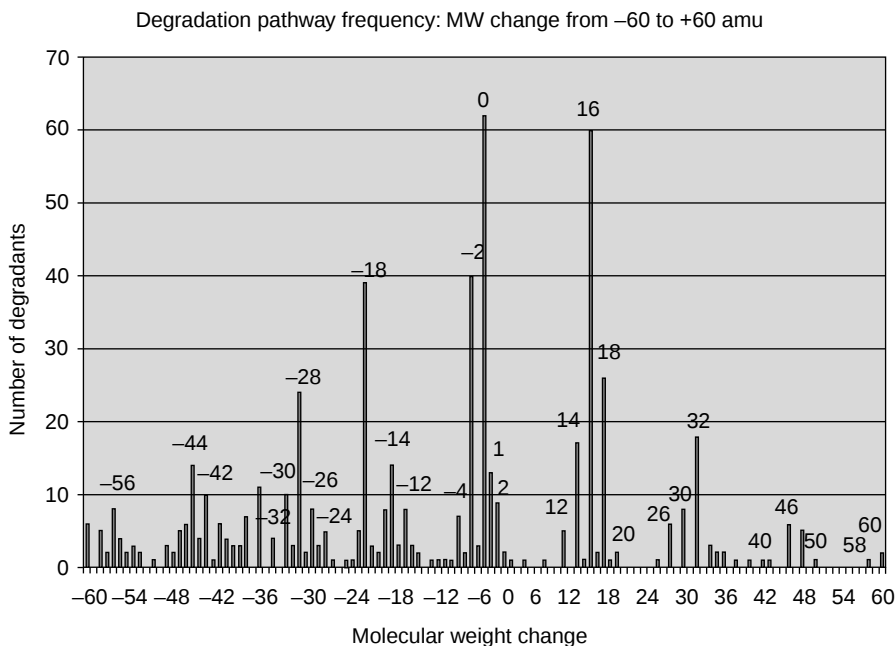


Figure 1 A plot of the frequency of specific MW changes from parent observed in degradants contained in the Pharma D3 database in 2009. The height of the specific bar graphs indicate the most frequent MW changes from parent as a result of drug degradation processes.

Table 1 Major Pathways Associated with Frequent Molecular Weight Changes (–60 to +60 amu)

MW Change	Number of Examples	Major Pathways Leading to MW Change
–44	14	Elimination of CO ₂ (14)
–42	8	Acetate hydrolysis-Ac (6) Acetamide hydrolysis-Ac (1) Dealkylation (1)
–34	7	Dechlorination (6) Dealkylation (1)
–30	10	–CO ₂ to ketone (8) –CH ₂ OH (1) Ar–NO ₂ to Ar–NH ₂ (1)
–28	20	–CO ₂ to –OH (9) –CO ₂ Et to –CO ₂ H (4) –NH ₂ Et to –NH ₂ (3) –B(OH) ₂ to –OH (1) –OEt to –OH (1) CO extrusion (1) i-Pr to carbonyl (1)
–18	28	–H ₂ O to double bond (8) Ar–Cl to Ar–OH (6) –H ₂ O to lactam (6) β-Lactam rearrangement (3) di-Hydropyridine to Ar–NO (2) R–Cl to R–OH (2) Seven other diff. transformations
–14	15	–CO ₂ Me to –CO ₂ H (6) R ₂ NMe to R ₂ NH (4) ArOMe to ArOH (1) Four other different transformations
–4	7	–2H ₂ and rearrangement (4) –H ₂ and C–OH to C=O (1) Pyrrolidine to pyrrole (1) Cyclohexene to benzene (1)
0	62	Epimerization/Racemization (31) <i>Cis/Trans</i> isomerization (8) Double bond migration (5)
1	11	–C=NH(NH–) to –C=O(NH)– (6) –CONH ₂ to –CO ₂ H (3) Rearr. to quat. Ammon. salt (1) 2–NH ₂ –pyridine to 2–OH–pyridine (1)
2	8	–CH ₂ /+O (3) Five other different transformations
14	17	PhCH ₂ –R to PhCO–R (11) R ₂ N–CH ₂ –R to R ₂ N–CO–R (3) Pyrrole to pyrrolidinone (1) R ₂ NH to R ₂ NMe (1) C=CH–CH ₂ – to C=CH–CO– (1)
16	60	<i>tert</i> -amine to N-oxide (13) Thio-ether to sulfoxide (6) Sulfoxide to sulfone (4) Allylic oxidation (3) Oxidation alpha to N or O (3)
18	23	Ester hydrolysis (6) Amide hydrolysis (4) Imine hydrolysis (4) β-Lactam hydrolysis (3) Imide hydrolysis (2) Four other different transformations

(Continued)

Table 1 (Continued) Major Pathways Associated with Frequent Molecular Weight Changes (–60 to +60 amu)

MW Change	Number of Examples	Major Pathways Leading to MW Change
32	18	Oxidation +2 oxygen atoms (5) Hydroperoxide formation (3) Sulfone formation (3) Pyrrole oxidation (2) Five other different transformations
46	5	Aminocyclopropane oxidation (1) Imidazole oxidation (1) Lactone to ethyl ester +EtOH (1) Alkene oxidation +MeOH (1) Pyrrole oxidation +MeOH (1)
48	5	Pyrrole oxidation +MeOH (2) Aminocyclopropane oxidation (1) heterocycle oxidation (1) Alkene oxidation +MeOH (1)
58	1	Reaction of hydroxyl or amine in parent with residual chloroacetic acid in sodium starch glycolate

source of the degradation was reaction of the parent drug with residual chloroacetic acid in sodium starch glycolate; had the Pharma D3 been available and populated at the time of these investigations, much time and expense could have been saved.

It is in this spirit of developing the field of degradation chemistry that this chapter is being written, in the hopes that the public and private knowledge base will grow rapidly, contributing to reducing the costs of developing new drugs and speeding up the development process.

Degradation of Common Functional Groups

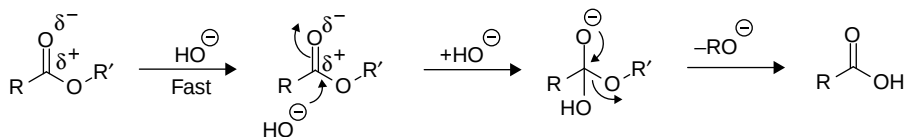
The degradation chemistry of a variety of common functional groups, organized by functionality, is discussed in this chapter. Some discussion of photolytic chemistry of functional groups is provided, but for a more thorough discussion of photochemistry (see chap. 7). This list of functional groups is not intended to be exhaustive, nor is the discussion intended to be comprehensive, as there exists volumes of reference books on the chemistries associated with various functional groups and heterocycles. For example, “The Chemistry of Functional Groups” series was founded by Professor Saul Patai (1918–1998) and in 42 years has published more than 130 volumes covering all aspects of organic chemistry (8). The discussion in the present chapter is intended to provide a quick and practical reference to the degradation chemistry of some of the more common functional groups found in the structures of typical active pharmaceutical ingredients (APIs).

Carbonyl Chemistry

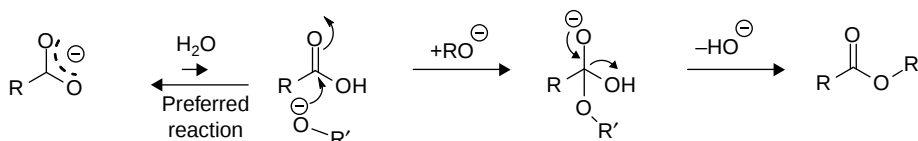
Esters, Lactones

While not particularly susceptible to oxidation, esters and lactones are subject to general acid or base-catalyzed hydrolysis to form a carboxylic acid and an alcohol (Fig. 2). Both are equilibrium reactions and readily occur when equilibrium is shifted to the right, although base-catalyzed hydrolyses are generally not reversible. Base-catalyzed hydrolysis (saponification) of esters is faster with the more powerful attacking hydroxide nucleophile (OH^-) yielding the salt of the acid. Acid-catalyzed ester hydrolysis is slower. Acids catalyze the reaction making the carbonyl carbon more positive (by protonation of the carbonyl oxygen) and therefore more susceptible to nucleophilic attack. Lactones are simply cyclic esters, and are therefore subject to

Base catalyzed hydrolysis of an ester and the reverse reaction



Reverse reaction (unlikely to occur, deprotonation of carboxylic acid is preferred)



Acid and base catalyzed hydrolysis of a lactone

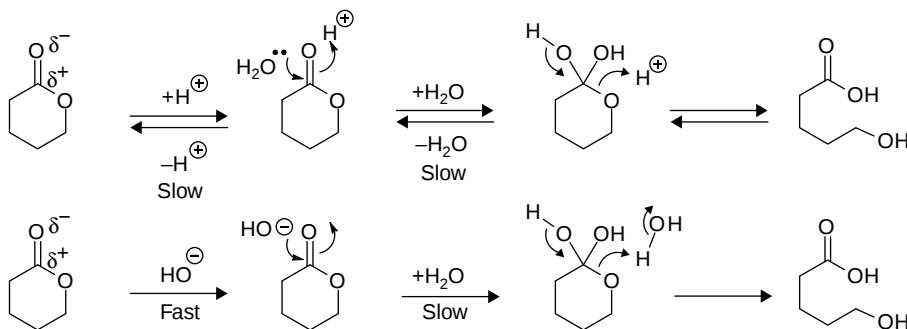


Figure 2 Hydrolysis mechanisms of esters and lactones.

the same acid/base hydrolysis chemistry as an ester (Fig. 2). The ring strain inherent in lactones tends to affect a larger degree of lability resulting in more facile/faster hydrolysis reactions.

Acid and base-catalyzed degradation can be highly influenced by changes in the concentrations of reagents present in the reaction. A reversible chemical reaction will achieve an equilibrium state, where the rates of formation of the products will be in balance with the rate of formation of the starting material. Le Châtelier's principle asserts that a change in the chemical system will cause the system to establish a new equilibrium. This principle may be invoked to explain some differences between degradation experiments performed in aqueous solutions and solid-state degradation. The presence of water in solution state degradation experiments will perturb the reaction by the presence of water in excess, which drives the equilibrium to form the hydrolysis products. However, removal of water would drive the reaction to undergo the reverse reaction to the ester or lactone. For example, Figures 2 and 3 illustrate the reversible nature of this hydrolysis reaction; removal of water would perturb the equilibrium and allow the reaction to form the lactone. This would be more likely to occur in solid-state degradation reaction conditions, where water levels are typically low.

The carbonyl carbon of these moieties is electrophilic, and therefore nucleophilic reactions are likely to occur via nucleophilic attack at the carbonyl carbon. In highly aqueous systems, the nucleophile is usually water. In the case of lactones, the smaller the lactone ring the

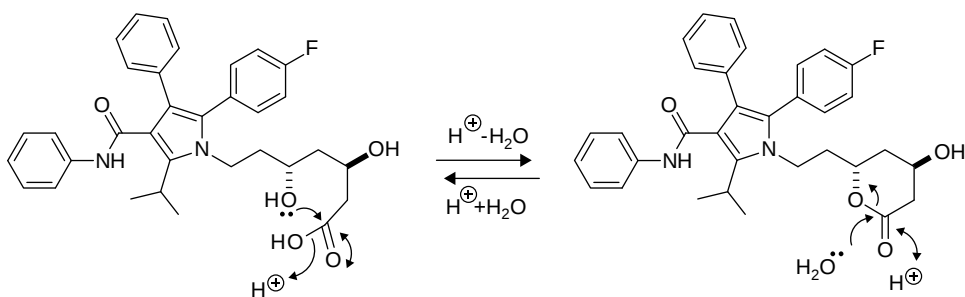


Figure 3 Reversible reaction of Lipitor with water under acidic conditions (9).

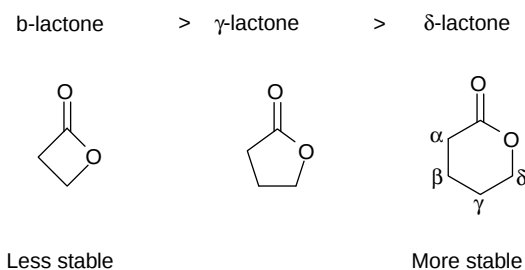


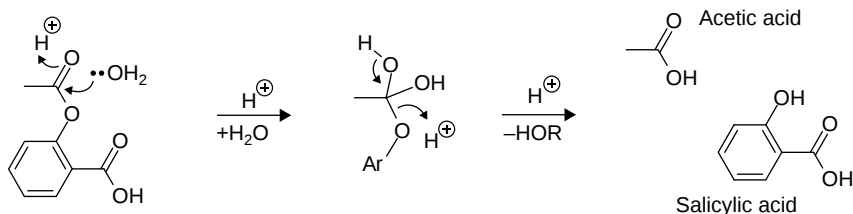
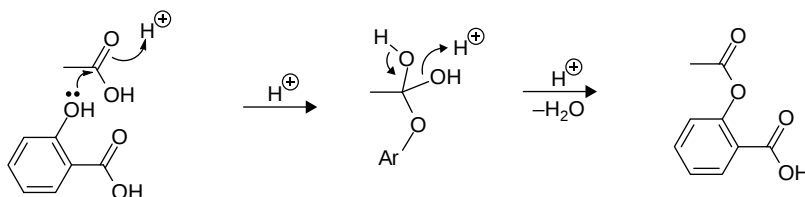
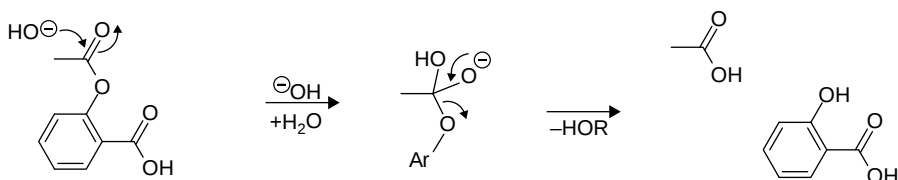
Figure 4 Relative rates of hydrolysis of lactones.

higher the ring strain, and therefore the more susceptible to hydrolysis (rate of hydrolysis of β -lactone $>$ γ -lactone $>$ δ -lactone) (Fig. 4).

For a more thorough discussion of acid/base hydrolysis, see Stewart and Tucker (2) March (10), and Mabey (11,12). A classic example of ester hydrolysis is demonstrated with aspirin. Aspirin hydrolyzes under acidic and basic conditions to yield acetic and salicylic acid (Fig. 5) (13). Basic hydrolysis tends to be much faster (9,10) than acid catalyzed hydrolysis because the hydroxide anion is a strong nucleophile and reacts directly, whereas a proton has to “find” a basic atom to interact with and “wait” for a water molecule (inherently less nucleophilic than hydroxide) to come close to the carbonyl carbon. Aspirin easily hydrolyzes because it is an activated ester (i.e., the leaving group can readily stabilize the anionic charge).

An additional API example that undergoes ester hydrolysis is cyclandelate (Fig. 6) (14). We can see that the presence of water in increasingly higher concentration will drive the reaction toward the product side of the equilibrium. Consequently, if water is removed and alcohol (i.e., R-OH) is added to the reaction mixture, a *trans*-esterification will occur, where R-OH (being in excess) can react and replace the substituted cyclohexanol with R-O to form a new ester. Hence, reversible reactions are important in degradation chemistry, especially when rationalizing the transition from solution state chemistry (high concentrations of water) to solid state (low concentrations of water). The base-catalyzed reverse reaction is much less likely to occur because the deprotonation of the carboxylic acid to form the anion is the dominant reaction, and it is difficult for a hydroxide anion to approach a negatively charged carboxylate anion because of the electrostatic repulsion of like charges.

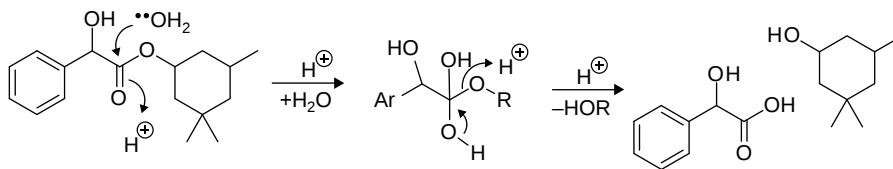
The API testolactone will undergo ring opening of the lactone to Δ' -testolic acid in strongly alkaline solution (Fig. 7) (15). Another example of lactone hydrolysis includes the API lovastatin (16). In addition to lactone *cleavage* reactions, lactone *formation* also occurs as in the case of the cefuroxime sodium (Fig. 8) (17).

Acid catalyzed hydrolysis (+H₂O)Acid catalyzed ester formation–reverse reaction (–H₂O, +ROH)Base catalyzed hydrolysis (+H₂O)**Figure 5** Hydrolysis of aspirin to acetic acid and salicylic acid.*Amides, Lactams*

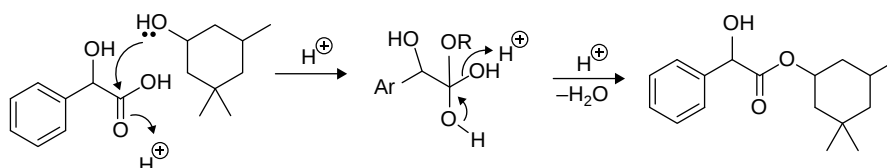
Amides are subject to acid or base-catalyzed hydrolysis to form a carboxylic acid and an amine (Fig. 11). Amides are more stable than their corresponding esters since -NHR is a poorer leaving group than -OR for esters. Therefore, water alone is not sufficient to hydrolyze most amides at a significant rate. Prolonged heating is also required even with acidic or basic catalysts. However, in pharmaceutical degradation studies, where we are not monitoring for stoichiometric chemistry, this reaction is often seen at levels of concern upon long-term storage at room temperature. Thioamides are much more readily hydrolyzed than amides. The rate of hydrolysis of thiol esters, esters, and amides (thiol esters > esters > amides) (Fig. 9) is a reflection the $\text{p}K_{\text{a}}$ of the conjugate acid of the leaving group (i.e., the thiol, the hydroxyl, and the amine leaving groups). The greater the tendency of the conjugate acid of the leaving group to ionize to the anion (i.e., the lower the $\text{p}K_{\text{a}}$ of the leaving group), the better the leaving group and the faster the hydrolysis (8).

The increased stability of amides with respect to esters is also due to partial delocalization of the nitrogen lone pair of electrons into the carbonyl group of the amide (Fig. 10). Addition of an nucleophile such as water is made more difficult if the electrophile is electron rich, because of electrostatic repulsion. This delocalization effect is also responsible for forming atropisomers, or “rotamers.” A rotamer is defined as a conformational isomer observable because of restricted rotation about a single bond. Sometimes the restricted rotation is sufficient to afford two distinct isomers that are easily resolved and detected by NMR; however, HPLC analysis will typically reveal only one distinct peak. It is not uncommon, however, especially when the R group on the nitrogen of the amide is large or bulky, for partial resolution of the rotamers by HPLC. The isomers are observed in NMR because the NMR time scale is comparable to the time scale of rotation about the single bond.

Acid catalyzed hydrolysis (+H₂O)



Acid catalyzed ester formation–reverse reaction (–H₂O, +ROH)



Base catalyzed hydrolysis (+H₂O)

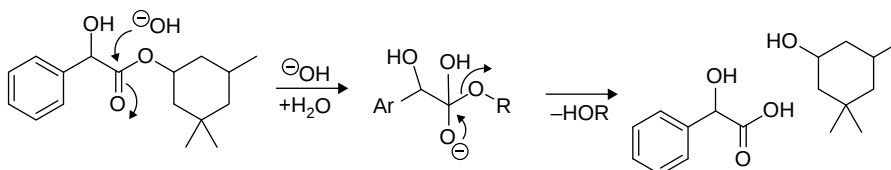


Figure 6 Hydrolysis of cyclandate and the reverse reaction under acidic conditions. (Base-catalyzed formation of the ester is not expected as discussed in the text.)

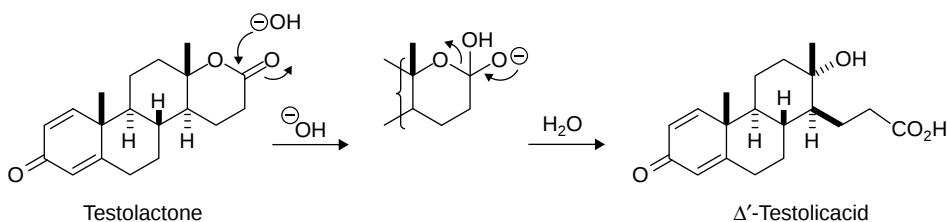


Figure 7 Testolactone degradation under basic conditions.

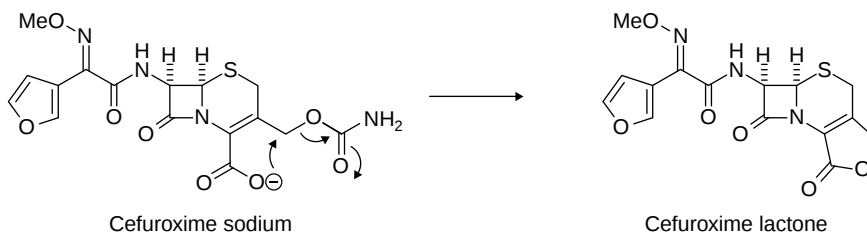
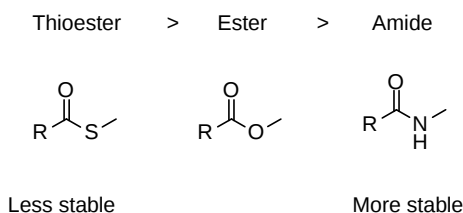
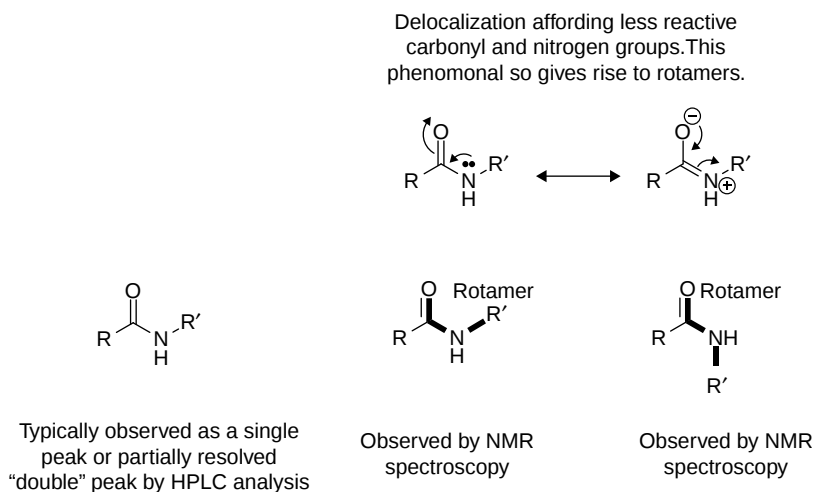


Figure 8 Cefuroxime lactone formation.

**Figure 9** Relative rates of hydrolysis.**Figure 10** Amide "Rotamers."

Lactams, imides, cyclic imides, and acyl-hydrazines also tend to undergo hydrolysis. Amides and lactams are not particularly susceptible to hydrolysis (Fig. 11).

Acetaminophen is a classic example of an API that readily undergoes amide hydrolysis. Acetaminophen undergoes acid and base-catalyzed hydrolysis to yield *p*-aminophenol and acetic acid (Fig. 12) (18).

Other examples of APIs that undergo amide hydrolysis examples include chloramphenicol (19), indomethacin under alkaline conditions (20), lidocaine (21), azintamide (22), terazosin (23), flutamide (24) oxazepam, and chlordiazepoxide (25). Lidocaine does not readily hydrolyze in aqueous solution under elevated temperatures under neutral to basic conditions (Fig. 13) (26). The enhanced hydrolytic stability of lidocaine is due to the steric hindrance of the two *o*-methyl groups; the two methyl groups on the phenyl ring of lidocaine are extremely close to the site of potential hydrolysis making the approach of the hydroxide anion difficult, resulting in "steric hindrance" of the hydrolysis. Hydrolysis does occur more readily in acidic conditions rather than basic conditions because the steric crowding does not affect the approach of the very small proton. Base catalyzed hydrolysis of amides containing an $-\text{NH}-\text{CO}-$ group is also inhibited by a competing deprotonation. Deprotonation of the amide affords a negatively charged anion that will electrostatically repel a "like" negatively charged anion.

Other APIs have shown lactam formation. In the case of baclofen, formation of the corresponding lactam is observed at 50°C (Fig. 14) (27).

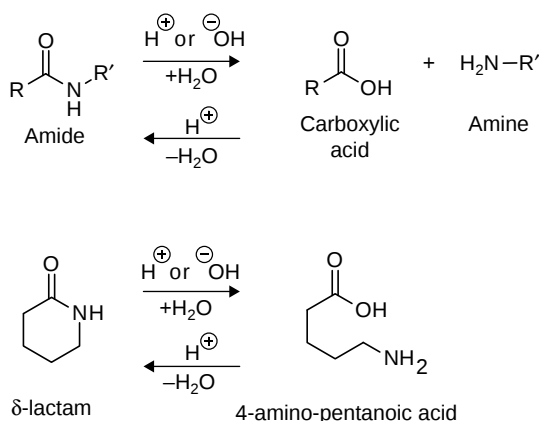


Figure 11 Hydrolysis of amides and lactams.

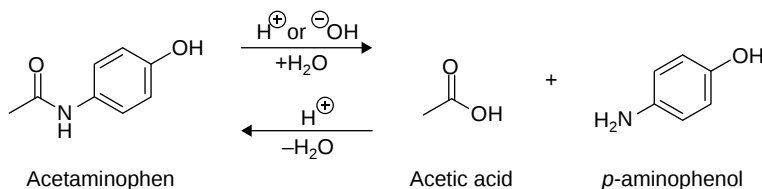


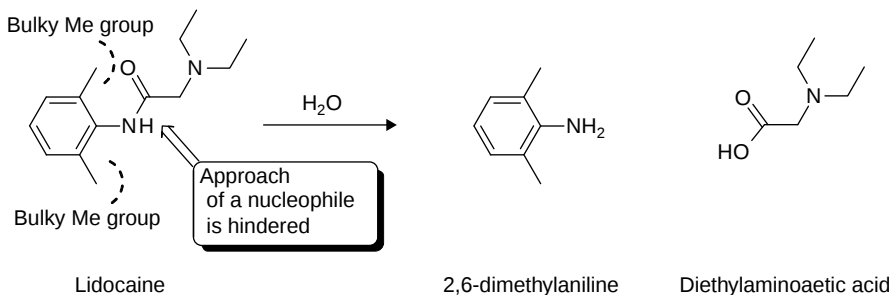
Figure 12 Acid and base catalyzed hydrolysis of acetaminophen.

A subset of lactams is the β -lactam functionality, the chemistry of which has been studied extensively (28,29). The β -lactam functionality has been thoroughly studied because the biological activity of β -lactam antibiotics (e.g., penicillins, cephalosporins, etc.) is the result of the presence of the β -lactam moiety (Fig. 15), which reacts with certain “penicillin binding proteins” found in bacteria to form a covalent bond (ester-linked) with the protein (30,31). The protein is thereby inactivated, bacterial cell wall/protein synthesis can no longer continue. The penicillin “caps” the active growing end of the growing protein strand, causing growing bacteria cells to undergo lysis. It is noteworthy that when a β -lactam undergoes hydrolysis, the initially formed product (Fig. 15) is generally not stable and undergoes further degradation to other products.

The electrophilic carbonyl of the β -lactam can also result in degradation by polymerization (32) as observed in the polymerization of ampicillin (33) (illustrated in Fig. 16), although not all polymerization reactions of β -lactams occur directly from a nucleophilic attack on the β -lactam [see for example, ceftazidime (34)]. In general, the polymerization of β -lactams occurs in a nonoxidative intermolecular condensation reaction, as is shown in Figure 16. The β -lactam moiety is not particularly susceptible to oxidation, but the sulfur atom in these antibiotics is susceptible to oxidation to the sulfoxide and sulfone (for more information on sulfoxide/sulfone chemistry see the sulfonyl chemistry section).

β -Lactam containing APIs are susceptible to lactam hydrolysis as observed with the β -lactam antibiotic penicillin G (23). Amoxicillin degradation is typical of penicillin hydrolysis reactions. Under basic conditions, amoxicillin decomposes by ring opening of the lactam ring to penicilloic acid, which ultimately loses CO_2 and forms penilloic acid (Fig. 17) (35). A general scheme for penicillin degradation is available in *Analytical Profiles of Drug Substances* (36).

Inhibition of hydrolysis by steric hinderance



Inhibition of base hydrolysis by competing deprotonation

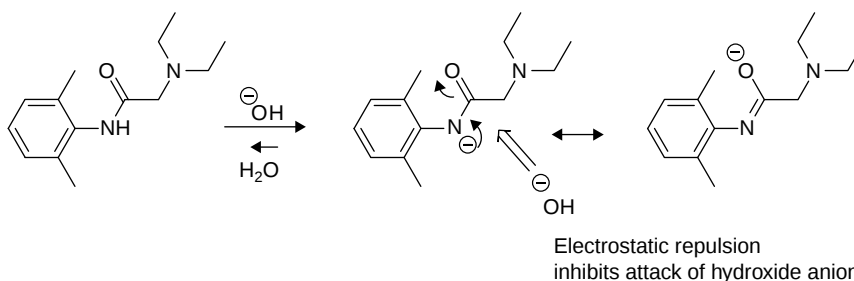


Figure 13 Lidocaine API amide hydrolysis and competing deprotonation under basic conditions.

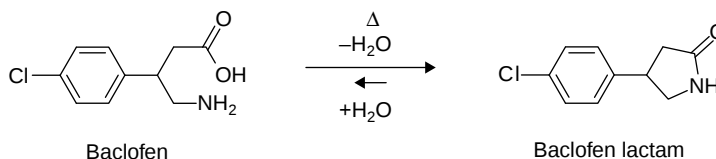


Figure 14 Degradation of baclofen to form baclofen lactam.

Carbamic Esters

Carbamic esters (carbamates) can also hydrolyze by the same chemistry described above for hydrolysis of esters and amides, to the corresponding carbamic acid, which under acidic conditions is followed by facile carbamic acid decarboxylation (Fig. 18). However, carbamates tend to hydrolyze more slowly than amides, typically making carbamate hydrolysis slow enough to be regarded as unlikely to occur under typical pharmaceutically relevant storage conditions (for solid oral dosage forms).

Example APIs containing carbamic ester functional groups with the potential for hydrolysis are loratadine and pipazetate (Fig. 19) (23).

Imides

Imides hydrolyze to give a mixture of products resulting from nucleophilic attack of water on either carbonyl carbon, as shown in Figure 20. In the case of cyclic imides such as maleimide, an intramolecular cyclization reaction can occur subsequent to the ring-opening hydrolysis,

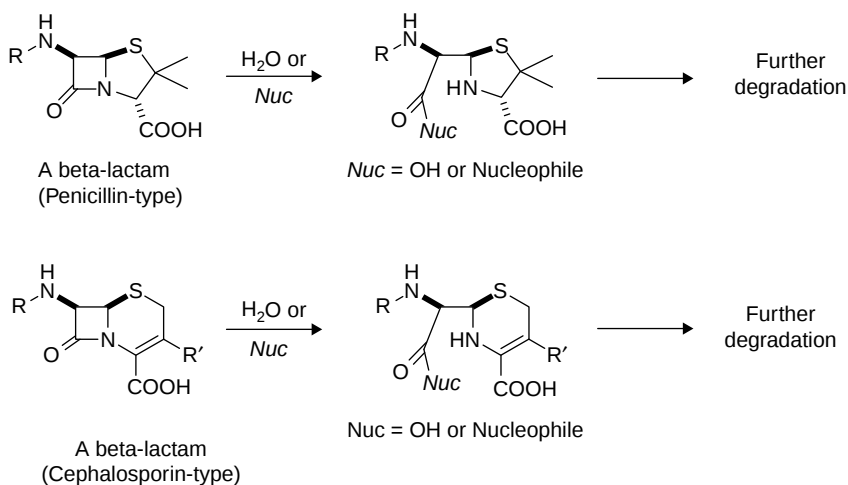


Figure 15 The highly electrophilic carbonyl carbon of β -lactam antibiotics reacts readily with nucleophiles.

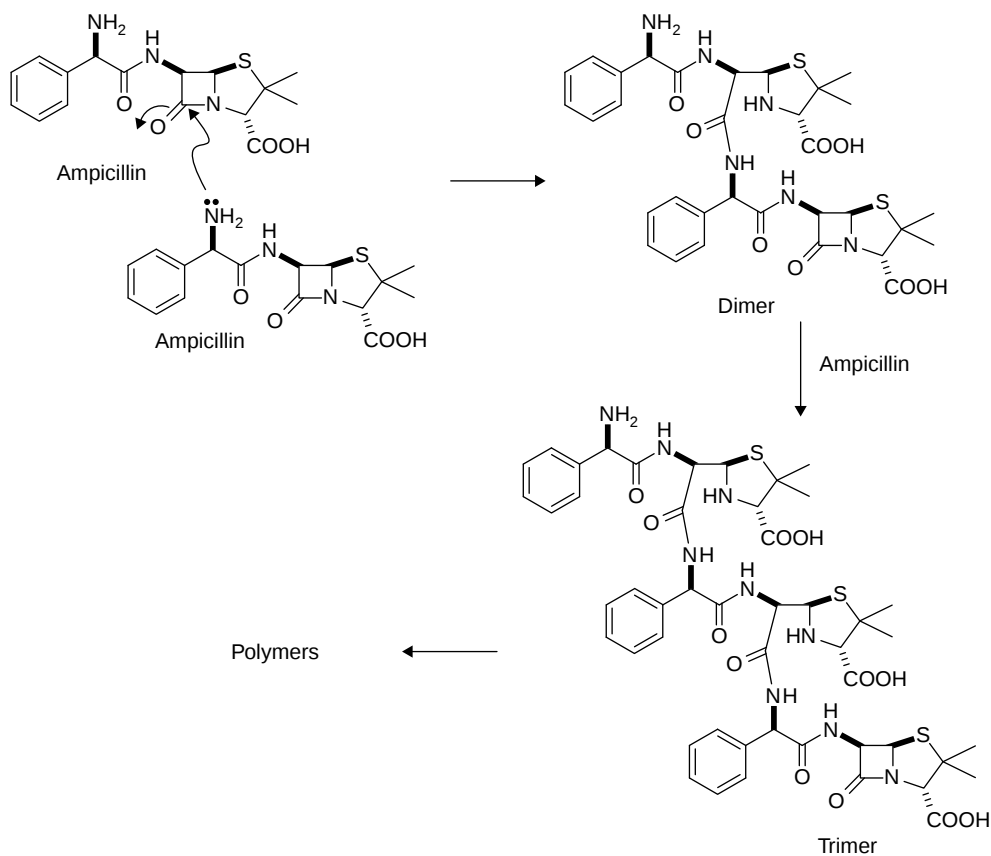


Figure 16 Polymerization of the β -lactam antibiotic ampicillin.

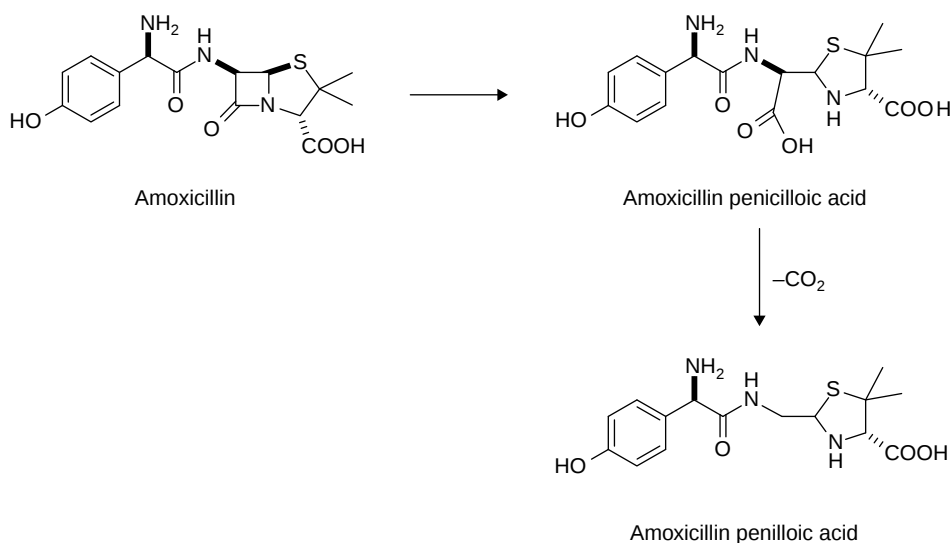


Figure 17 Hydrolysis and decarboxylation of the antibiotic amoxicillin.

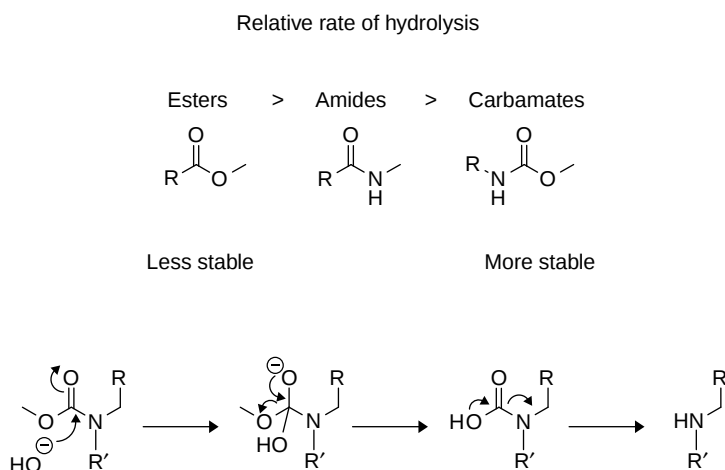


Figure 18 Carbamic ester hydrolysis.

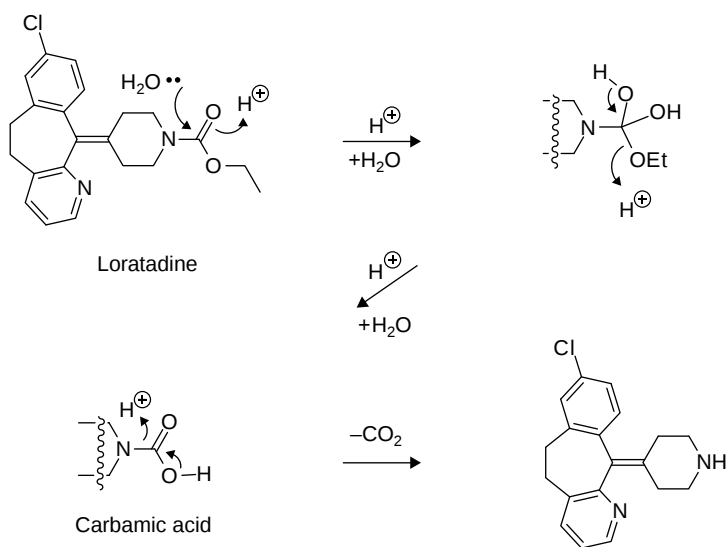
leading to the release of ammonia and the formation of maleic anhydride (37). Alternatively, the ring-opened product can hydrolyze further without ring closure to form maleic acid.

An example of imide hydrolysis occurs with the API glutethimide (Fig. 21). The mechanism proposed involves direct attack by a hydroxyl ion on the sterically less-hindered carbonyl followed by ring cleavage (38).

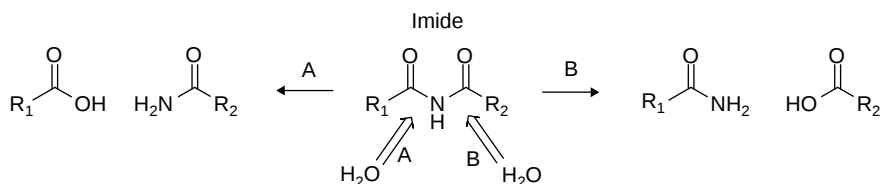
Additionally, imide hydrolytic decomposition is observed with the API phenobarbital in alkaline solution to produce α -ethylbenzeneacetic acid and urea (Fig. 22) (39).

Carboxylic Acids

Carboxylic acids *typically* have pK_a 's in the range of ~2–5.5 (although some can be significantly outside this range, depending on the nature of the substituents) (40) and it is therefore helpful to consider the ionization state of the group when evaluating the chemistry. Below the pK_a , the



Imide hydrolysis



Cyclic imide hydrolysis

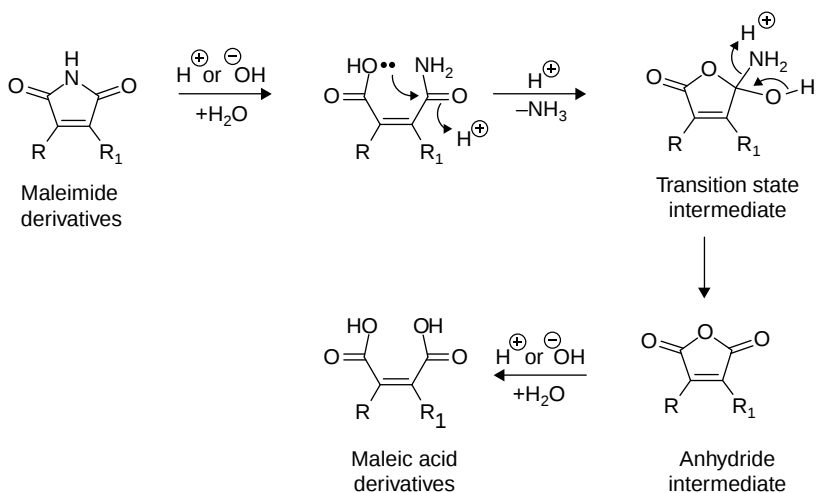


Figure 20 Hydrolysis of imides.

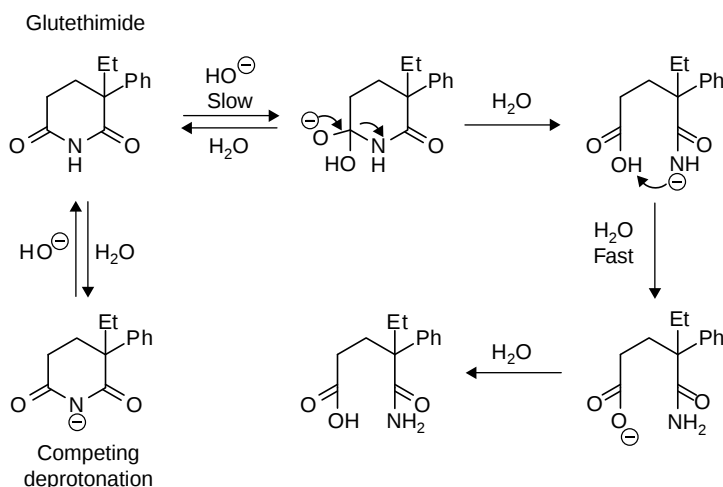


Figure 21 Mechanism of glutethimide degradation.

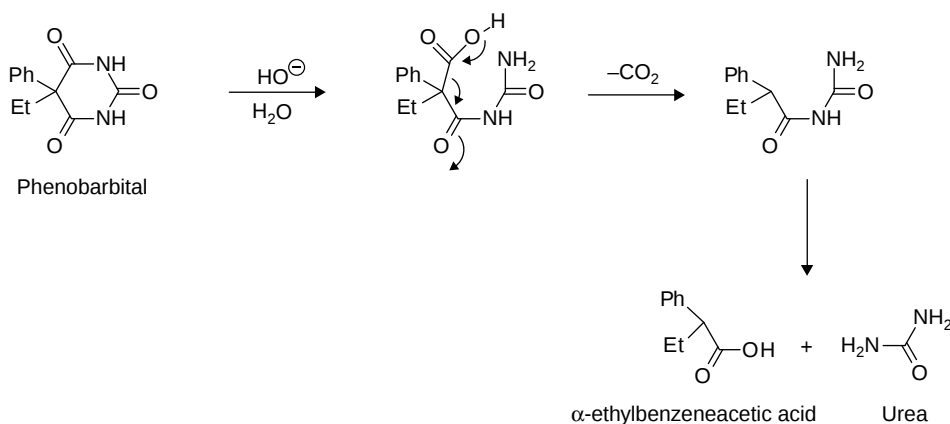


Figure 22 Imide hydrolysis of phenobarbital under basic conditions.

group is protonated and therefore the carbonyl carbon is more electrophilic. The electrophilic carbonyl can undergo nucleophilic attack to form esters, amides, thioesters, etc. In the case of attack by an alcohol, the reaction product is an ester, and the reaction is called an esterification reaction (Fig. 23). This can occur as an artifact reaction when acid/base hydrolysis reactions are performed using an alcohol cosolvent system such as methanol. Esters of the API parent compound can also be observed as process related impurities, especially when alcohol solvents are used in the recrystallization step.

If the pH is higher than the pK_a , the carboxylic acid will deprotonate resulting in the carboxylate (anionic) form, where the negative charge is resonance stabilized; this spreading of the high-energy anion across the three atoms of the carboxylate group result in a lower electron charge density and a relatively stable anion. The resultant anionic group is therefore less electrophilic than the carboxylic acid, and does not have a leaving group. Therefore, reactions with nucleophiles are significantly suppressed when compared to the protonated form; however,

carboxylates can act as a weak nucleophile. Carboxylic acids are not prone to oxidative degradation.

Some carboxylic acids can decarboxylate under right conditions (41). For example, if a carbonyl group is β to a carboxylic acid, acid or base-catalyzed decarboxylation can occur as shown in Figure 23.

As an example, the API moxalactam disodium undergoes decarboxylation (i.e., loses carbon dioxide) at the benzylic site to form decarboxylated moxalactam in the solid state (Fig. 24) (42).

Decarboxylation of the API diflunisal occurs under thermal conditions. Diflunisal does not appear to have a β -carbonyl (or other double bonded functional group) available to participate in a decarboxylation reaction; however, the phenol functional group can exist in a keto form via tautomerization. Low levels of this keto form provide the β -carbonyl needed to allow decarboxylation to the decarboxy degradant. Decarboxy diflunisal also has a hydroxyl

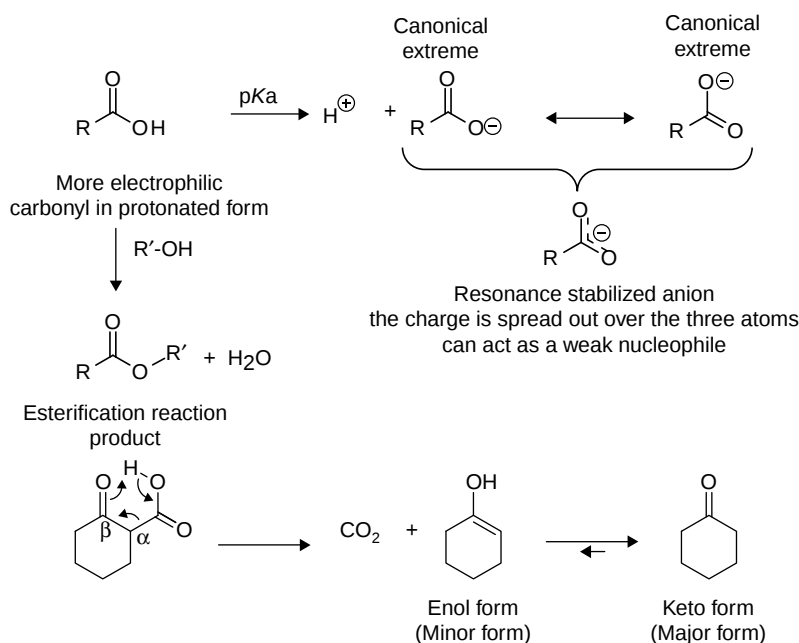


Figure 23 (Upper) Chemistry of carboxylic acids. (Lower) Decarboxylation of a β -keto carboxylic acid.

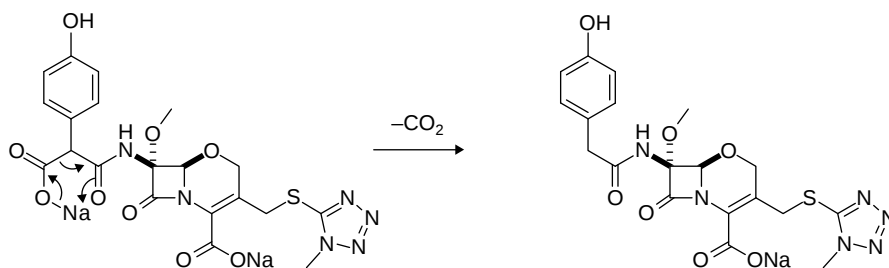


Figure 24 Moxalactam disodium decarboxylation.

functional group, and subsequently reacts with the carboxylic acid group of another molecule of diflunisal to produce the diflunisal decarboxydiflunisal ester (Fig. 25) (43). Other examples of API decarboxylation include norfloxacin (44) and terazosin (45).

An example excipient that is reactive with APIs containing carboxylic acids is polyvinyl alcohol. Polyvinyl alcohol containing secondary hydroxyl groups is susceptible to esterification reactions (46). Other excipient sources of hydroxyls that react with APIs containing carboxylic acids are carbohydrates/sugars such as lactose, mannitol, sucrose, β -cyclodextrins, and polyethylene glycols (47).

Ketones, Aldehydes

Ketones and aldehydes have electrophilic carbonyls that significantly contribute to the chemistry of these functional groups. In general, aldehydes tend to be more electrophilic and will often exist in aqueous solutions in hydrated form as a gem-diol (Fig. 26). This is an important

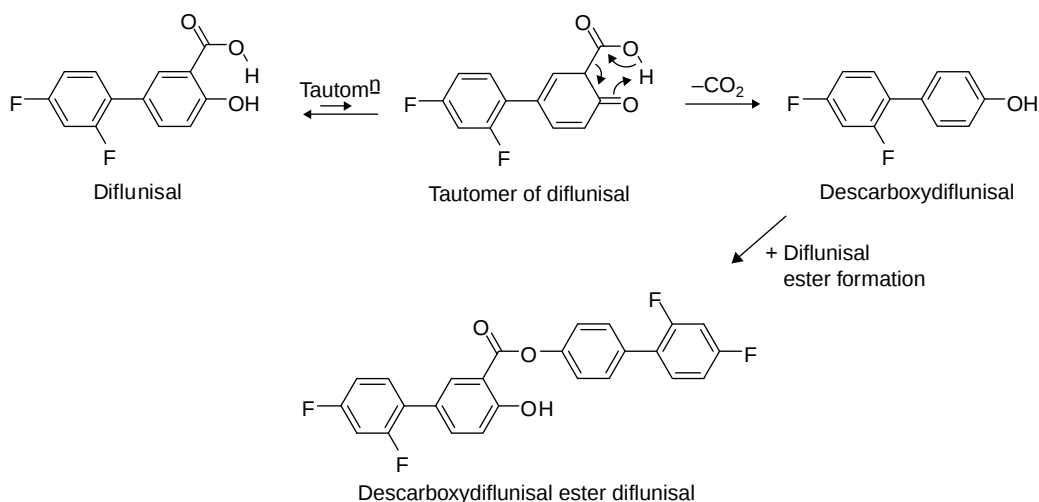


Figure 25 Diflunsal decarboxylation and dimer ester formation.

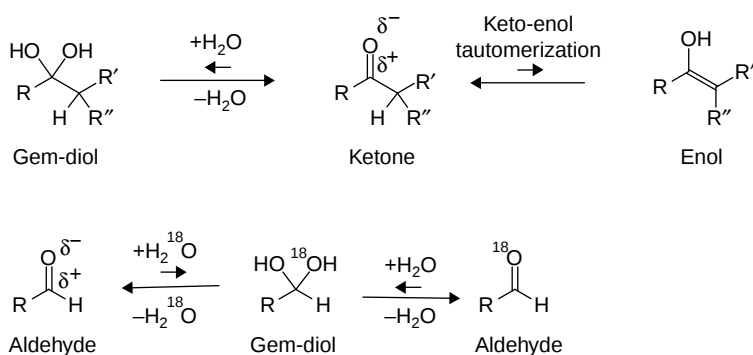


Figure 26 Chemistry of ketones and aldehydes.

consideration when attempting to characterize by NMR an unknown that may contain an aldehyde, since in D_2O the aldehyde might exist predominantly as a gem-diol but in organic solvents such as $CDCl_3$ the keto form will likely predominate. Isolated ketones may also readily react with water to form a gem-diol, although generally to a much lesser extent (48). Ketones (and aldehydes) will undergo a rapid tautomerization (catalyzed by either acid or base) known as the keto-enol tautomerism, if there is a hydrogen atom on the carbon alpha to the carbonyl. Thus, chiral centers adjacent to the carbonyl of ketones and aldehydes are often susceptible to epimerization via this tautomerization.

Aldehydes and ketones also react with water to form a gem-diol hydrate. This hydrated form of an aldehyde or ketone can be present in aqueous solutions in equilibrium with the carbonyl form. This equilibrium can be demonstrated and the rate of hydration can be measured with the use of ^{18}O isotopically labeled water. The rate of incorporation of ^{18}O into the molecule can be measured. It can therefore be problematic to use ^{18}O -labeled water or molecular oxygen to investigate the mechanism of formation of an aldehyde or ketone containing degradation product, due to this exchange.

Because of their significant electrophilic character, aldehydes are often unstable and will react with nucleophiles. For example, a common reaction of aldehydes is the formation of a hemiaminal with amines. If the amine is a primary amine, the hemiaminal can dehydrate to form an imine as shown in Figure 27. The reaction of aldehydes with primary and secondary amines is a well-studied reaction pathway because it is a common reaction pathway of reducing sugars and amino acids, and this reaction pathway is known as the Maillard reaction (49). In the case of amino acids and sugars, this reaction leads to discoloration, or "browning." This reaction will be discussed in greater detail in section "Amines–Maillard Reaction," later in this chapter.

Aldehydes are susceptible to oxidation to the corresponding carboxylic acid, but ketones are generally not oxidized under pharmaceutically relevant conditions. When ketones are conjugated with one or more double bonds, as in the case of an α,β -unsaturated ketone (also called an "enone"), the carbonyl is less electrophilic but is still susceptible to nucleophilic attack at either the carbonyl carbon (1,2-addition) or at the β -carbon (1,4-addition, or "Michael addition") (Fig. 28).

The carbon alpha to the carbonyl of aldehydes and ketones can act as a nucleophile in reactions with other electrophilic compounds, or intermolecularly with itself. The nucleophilic character is imparted via the keto-enol tautomerism. A classic example of this reactivity is seen in the aldol condensation (50) as shown in Figure 29. Note that the aldol condensation is potentially reversible (retro-aldol), and compounds containing a carbonyl with a hydroxyl at the β -position will often undergo the retro-aldol reaction.

The aldol condensation reaction is catalyzed by both acids and bases. Aldol products undergo a reversible dehydration reaction (Fig. 29) that is acid or base catalyzed. The dehydration proceeds through an enol intermediate to form the α,β -unsaturated carbonyl containing compound.

The API haloperidol was found to be incompatible with 5-(hydroxymethyl)-2-furfuraldehyde, an impurity in anhydrous lactose (resulting from the degradation of lactose), resulting in the formation of an adduct (Fig. 30) (51).

Aldehydes and ketones are known to be photoreactive functional groups (Fig. 31) (52). The absorption of a photon excites an electron from a ground state bonding orbital to a π^*

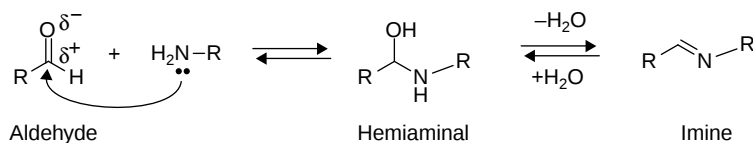


Figure 27 Reaction of aldehydes with amines.

antibonding orbital. The corresponding radical behaves as an electrophilic radical in the $n\pi^*$ excited state. Common reactions from this excited state include reduction to the alcohol via intermolecular hydrogen abstraction, fragmentation either via α cleavage (Norrish Type I) or via intramolecular γ -hydrogen atom abstraction followed by C–C cleavage (Norrish Type II).

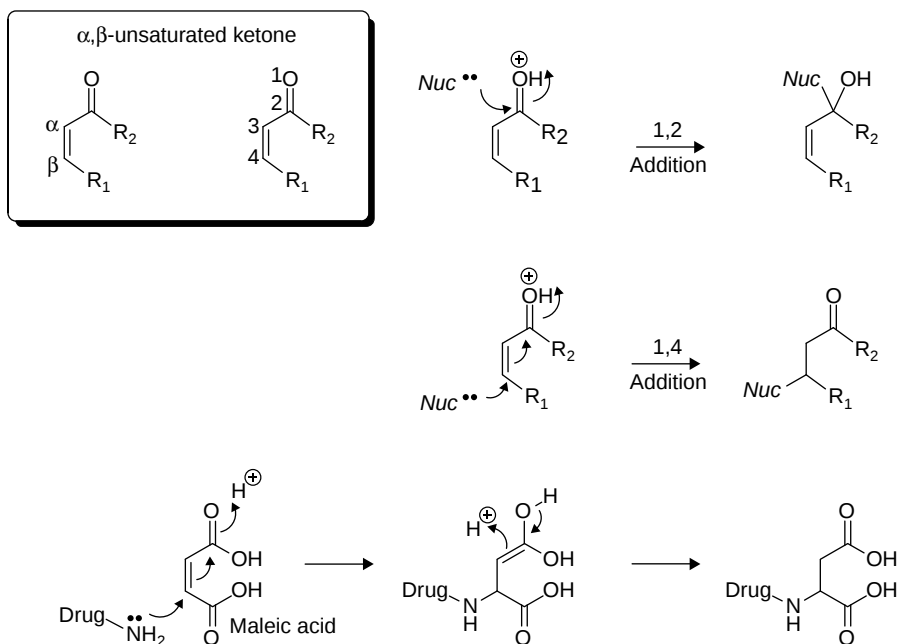
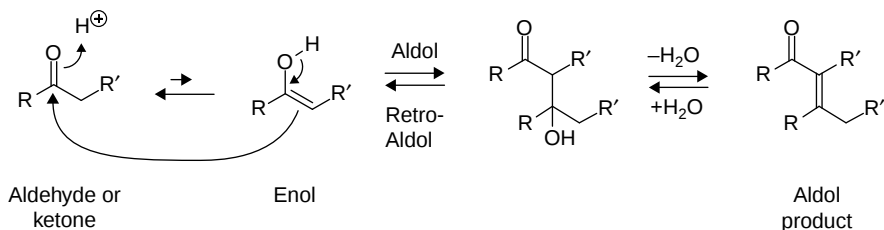


Figure 28 Reactions of α,β -unsaturated ketones with nucleophiles.

Aldol reaction (a ketone reaction with another ketone)



Aldol reaction (illustrated with formaldehyde; a common contaminant)

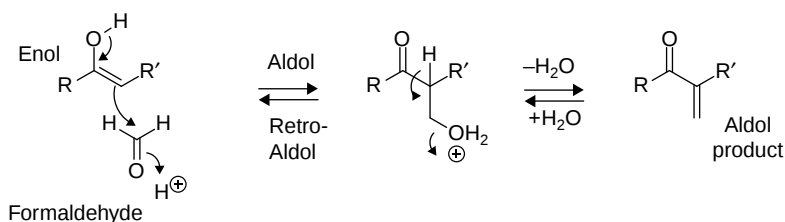


Figure 29 "Aldol" condensation.

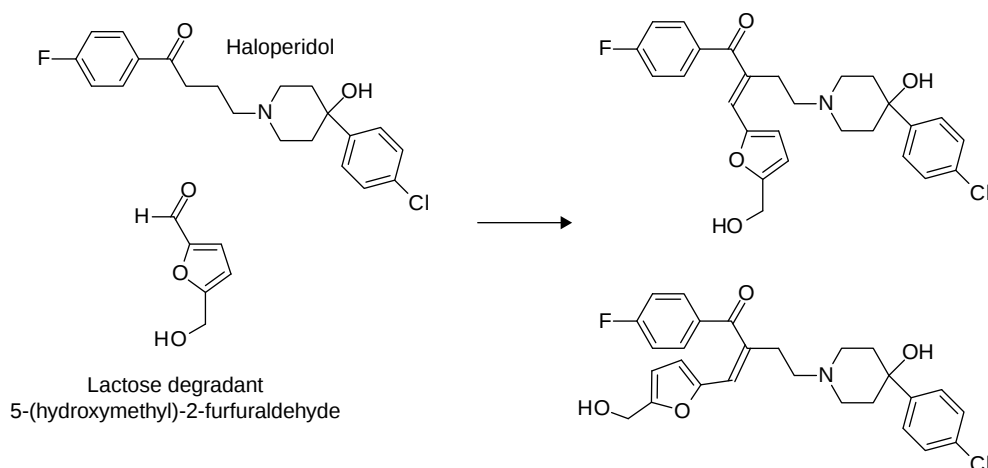


Figure 30 Haloperidol aldol condensation product.

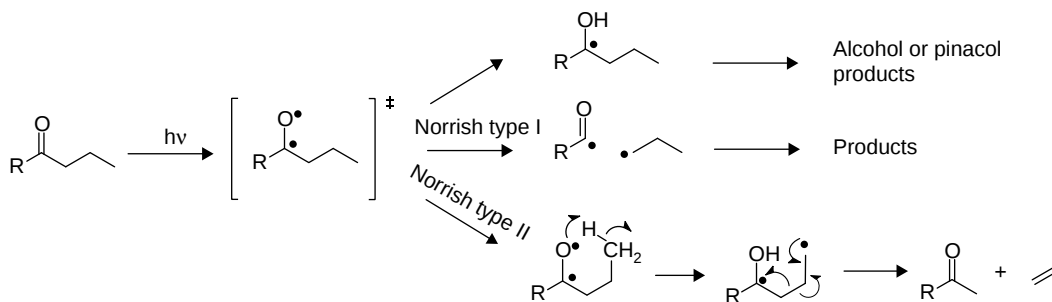


Figure 31 Aldehyde/ketone photochemistry.

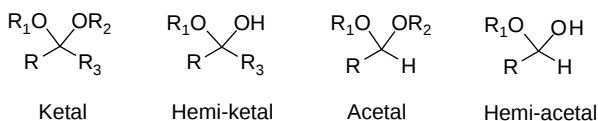


Figure 32 Basic structures of ketals, hemi-ketals, acetals, and hemi-acetals.

Acetals/Ketals

Hemi-acetal and hemi-ketal functional groups (Fig. 32) are susceptible to acid or base-catalyzed hydrolysis. For acetals and ketals, only acid-catalyzed hydrolysis occurs (23). Acetals and ketals are extremely resistant to hydrolysis by base. The acid-catalyzed reaction proceeds by an S_N1 mechanism as shown in Figure 33 for acetals.

Triamcinolone acetonide contains a cyclic ketal group that can be readily cleaved by a variety of organic acids (Fig. 34) (53).

Nitrogen Containing Functional Groups

Nitriles

Nitriles (Fig. 35) are susceptible to hydrolysis via nucleophilic attack of water on the electro-positive carbon atom, especially under strongly acidic and basic conditions. Nitriles hydrolyze to imidic acids, which tautomerize to amides. Amides can be hydrolyzed further to carboxylic

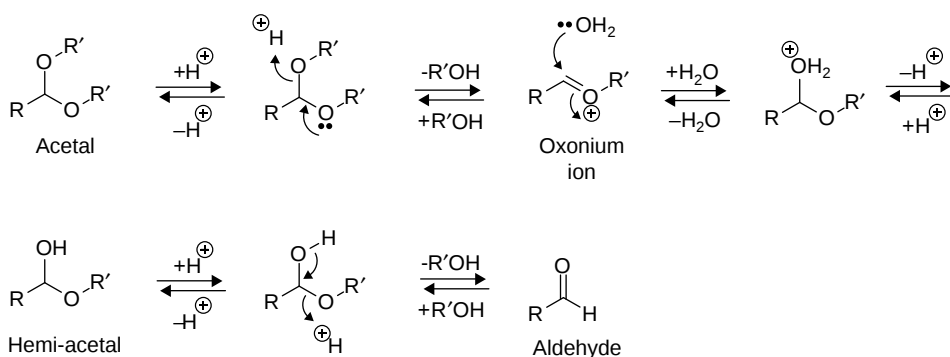


Figure 33 Acid-catalyzed (S_N1) acetal hydrolysis mechanism.

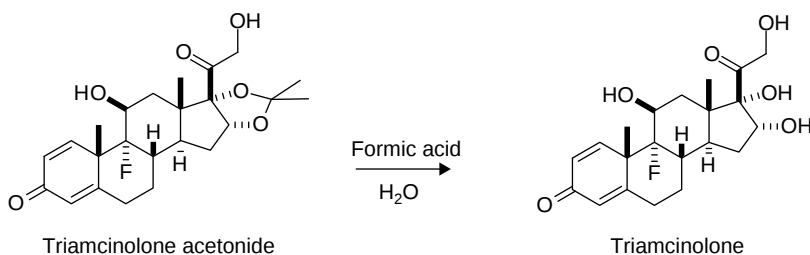


Figure 34 Triamcinolone acetonide ketal hydrolysis.

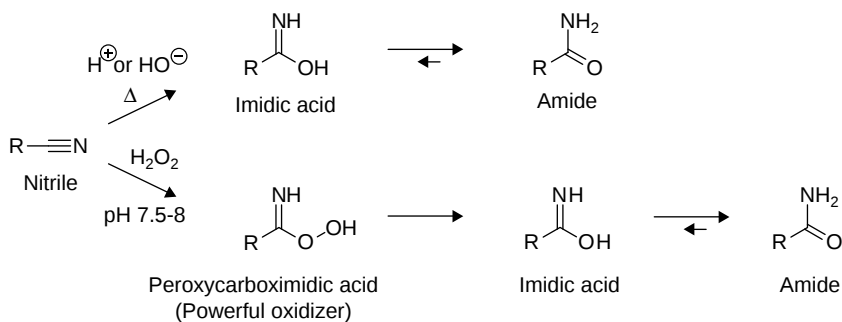


Figure 35 Hydrolysis and oxidation of nitriles.

acids, although much more slowly. Nitriles are susceptible to oxidation by peroxides under mildly basic conditions (e.g., pH 7.5–8) as has been documented in the case of acetonitrile (54,55). The nitrile adds peroxide to form an unstable peroxycarboximidic acid (123). This unstable intermediate is a highly reactive oxidizing species and can oxidize other species present while being concomitantly reduced to the amide (Fig. 35).

Peroxy-carboximidic acid is a stronger oxidizer than hydrogen peroxide; hence, the use of acetonitrile as a co-solvent for hydrogen peroxide stress testing may produce unusually rapid oxidation with the possibility of an overly complex degradation profile when the pH of the solution is greater than 7. It is possible for peroxycarboximidic acid to decompose to form singlet oxygen (56).

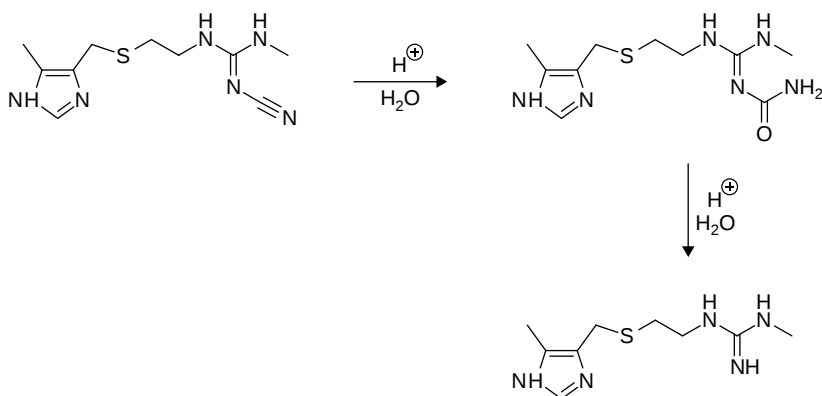
Acid hydrolysis of the nitrile in the API cimetidine leads to the corresponding “amide” through loss of the CONH_2 moiety via a pathway that is analogous to a β -keto acid decarboxylation (Fig. 36) (57).

If the carbon alpha to the nitrile contains a proton, the potential exists for radical-initiated oxidation, leading to further oxidative degradation. Such reactivity has been observed with acetonitrile (Fig. 37) (58,59,60) contrary to the general perception of acetonitrile as an inert solvent.

The API diphenoxylate hydrochloride undergoes degradation under acidic conditions with peroxide to hydrolyze the tertiary amine followed by ring closure as the hydroxyl group adds to the amide (hydrolyzed nitrile) group (Fig. 38) (61).

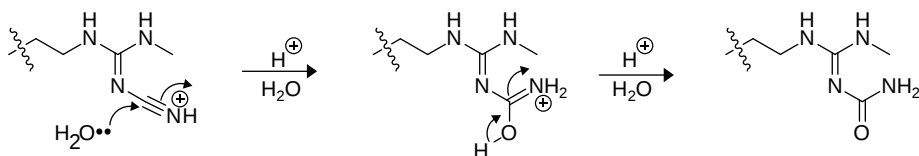
Amines

Amines are a very common functional group in pharmaceuticals and are prone to a variety of degradation reactions. Amines can be primary, secondary, or tertiary, aryl or alkyl. The protonation state of amines is critical to an understanding of the degradation chemistry. Most primary, secondary, and tertiary alkyl amines have pK_a 's in the range of 7.5–11.5. Aryl amines tend to be

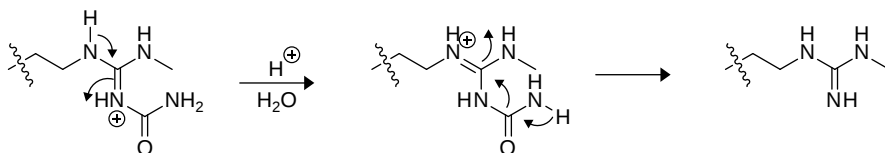


Proposed mechanism

First step protonation followed by hydrolysis of the nitrile.



Second step:



Similar to β -keto acid decarboxylation

Figure 36 Cimetidine hydrolysis.

much less basic and have pK_a 's in the range of 4–6 (e.g., pK_a of aniline is 4.6). When amines are unprotonated (i.e., in the neutral “free base” form), they are nucleophilic, more easily oxidized, and more volatile. Primary and secondary amines are nucleophilic and will react readily with electrophiles such as aldehydes (as present in excipients such as glucose, lactose, etc.) to undergo the first steps of the Maillard reaction (Figs. 39 and 52). Amines may also react with trace levels of formaldehyde (or other aldehydes adventitiously present) to form hemiaminals with the potential for dehydration to imines and/or cross-linking with other amines or nucleophiles as shown in Fig. 39. Such reactions of amines with formaldehyde have been documented

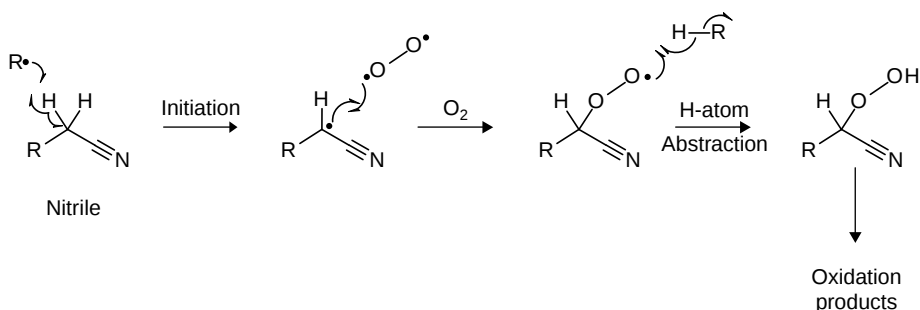


Figure 37 Potential autoxidation degradation pathway of nitriles.

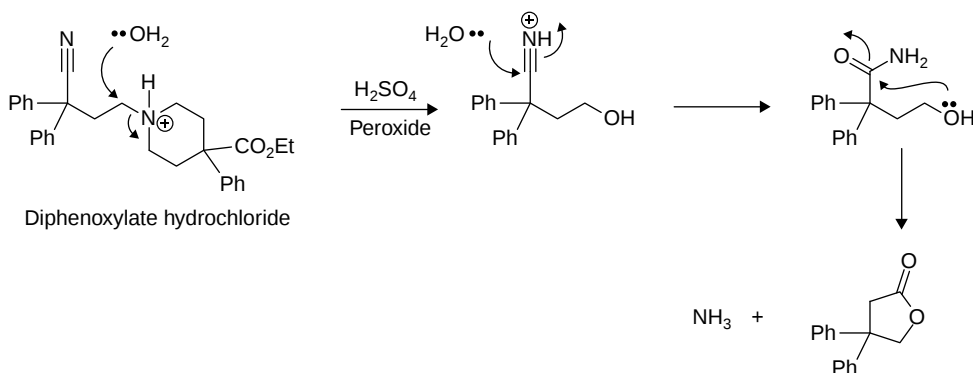


Figure 38 Diphenoxylate hydrochloride degradation under acid/hydrogen peroxide conditions.

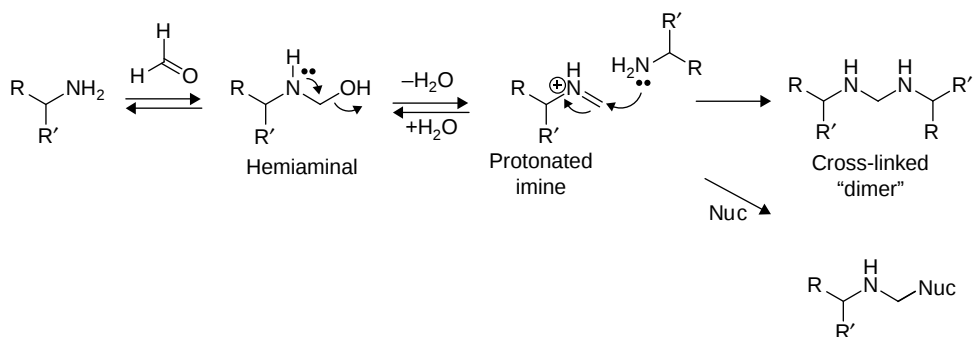


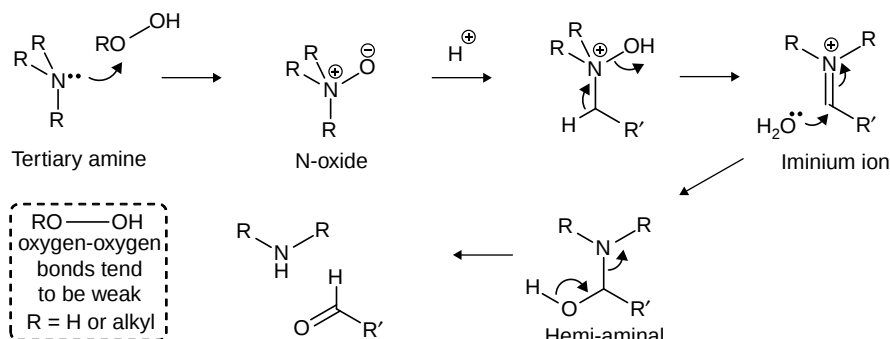
Figure 39 Reaction of an amine with formaldehyde.

in the literature and should be considered as possible degradation pathways (62,63,64). This chemistry is commonly observed in the degradation of formulated drugs. Excipients (65) especially carbohydrates and polymeric excipients containing ethylene glycol repeating units (e.g., polyethylene glycol and polysorbates) are a common source of formaldehyde from oxidative breakdown of the polymeric chain (66).

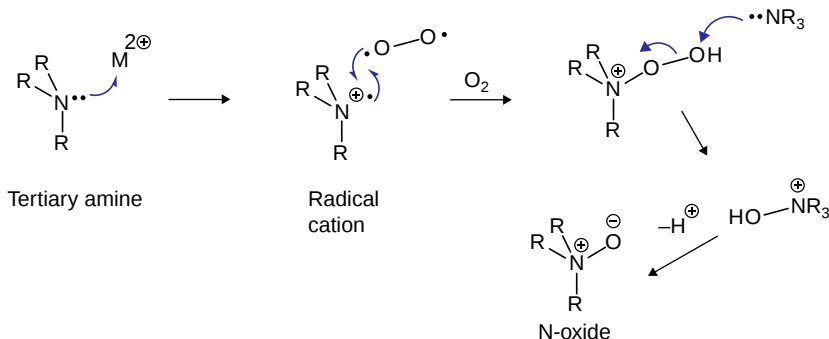
Tertiary amines are known for their propensity to oxidize to the amine oxide (*N*-oxide) during long-term storage (67) (Fig. 40).

A classic example of the oxidation of a tertiary amine to form an *N*-oxide is the case of raloxifene hydrochloride (64). The tertiary amine of raloxifene hydrochloride is protonated and

Oxidation of tertiary amines



Oxidation of tertiary amines (proposed alternative mechanism)



Oxidation of secondary amines

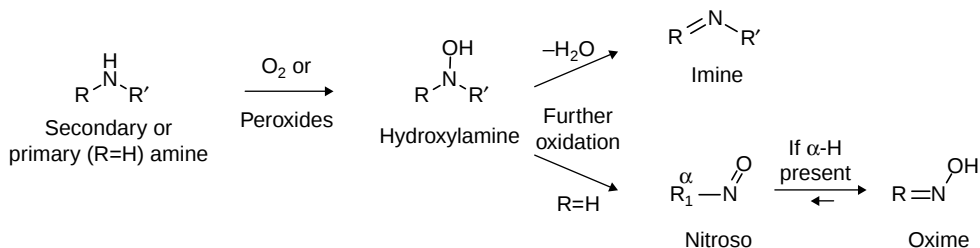


Figure 40 Formation of *N*-oxides from oxidation of tertiary amines and formation of hydroxylamines from oxidation of primary and secondary amines.

tertiary amines may still present problems for long-term storage or formulation, as was observed in the case of raloxifene hydrochloride (67). Oxidation of aryl amines leads to aryl hydroxylamines, which are susceptible to further oxidation to aryl nitroso compounds. Aryl nitroso compounds are genotoxic alerting structures (70).

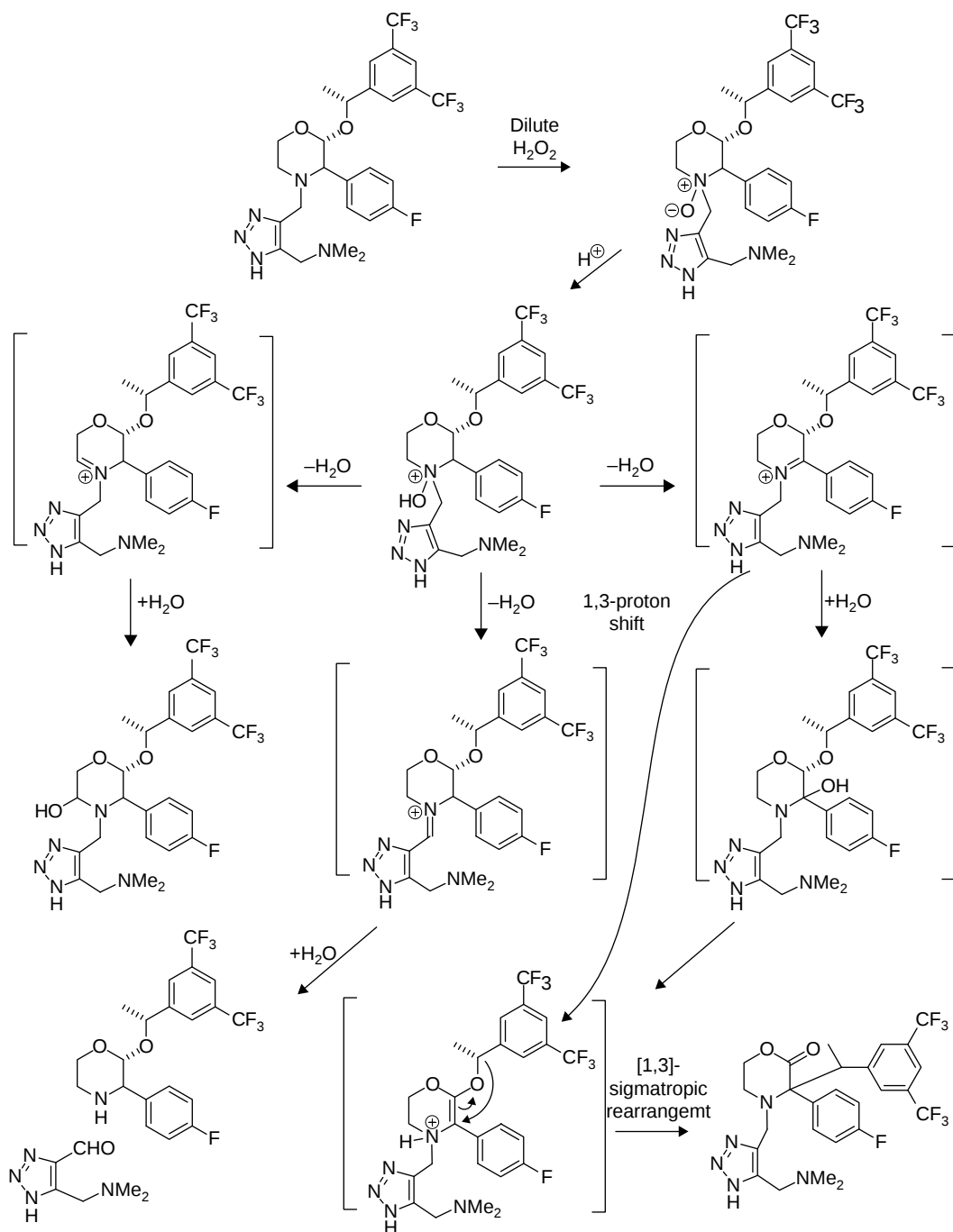


Figure 43 Proposed degradation pathways for an amine oxide degradation product. The counterion (chloride, Cl⁻) is not shown for simplicity.

Heteroaromatic amines can oxidize to the corresponding *N*-oxide, which are typically stable enough to be isolated and detected as degradation products. Aromatic *N*-oxides are genotoxic alerting structures (70). The *N*-oxide functionality typically increases the reactivity of the aromatic ring. For example, the *N*-oxide functionality in pyridine *N*-oxide facilitates both electrophilic and nucleophilic substitution at the alpha and gamma positions (71).

Aliphatic amines are subject to simple acid (Fig. 44) and base-catalyzed hydrolysis to the resulting hydroxyl compound or elimination to form a double bond. In either case, ammonia is eliminated from the API.

Using the peracid *m*-chloroperbenzoic acid (*m*-CPBA), the API dibucaine can be easily oxidized to its *N*-oxide analog (Fig. 45) (72). An additional example includes the API flavoxate hydrochloride, which degrades to the corresponding *N*-oxide in 3% aqueous hydrogen peroxide (73). The use of an oxidizing reagent such as *m*-CPBA to prepare *N*-oxides can be extremely useful in synthetically preparing degradants for identification and for the preparation of compounds as standards.

Dealkylation is also a common degradation reaction for amines (Fig. 46). As shown in the mechanistic scheme, dealkylation involves oxidation of the amine by peroxide followed by decomposition (dehydration) of the hydroxylamine to the corresponding imine. Water attack on the imine occurs to yield the corresponding primary amine and aldehyde. Amine dealkylation can also occur via radical-mediated oxidation processes, as shown in Figure 47.

As an example of a secondary amine dealkylation via oxidative degradation, the API brinzolamide undergoes a dealkylation reaction converting the secondary amine to a primary amine via peroxide mediated oxidation and elimination of water, followed by hydrolysis of the imine as shown in the presence of heat, light (neutral pH) and peroxide (Fig. 48). In the presence of peroxide, brinzolamide also undergoes oxidation to the corresponding hydroxylamine (74).

In the presence of light, dorzolamide hydrochloride also undergoes amine dealkylation from a secondary to a primary amine (Fig. 49), which presumably occurs via oxidation to the hydroxylamine, dehydration to the imine, and subsequent hydrolysis to form the primary amine (75).

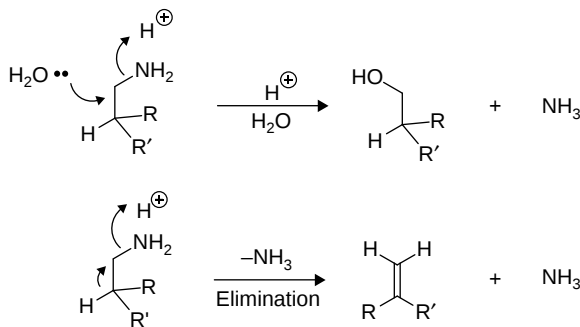


Figure 44 Acid-catalyzed hydrolysis of a primary aliphatic amine to the alcohol, or elimination to the alkene.

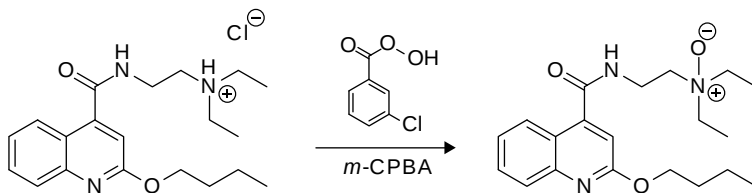


Figure 45 Dibucaine hydrochloride *N*-oxide formation.

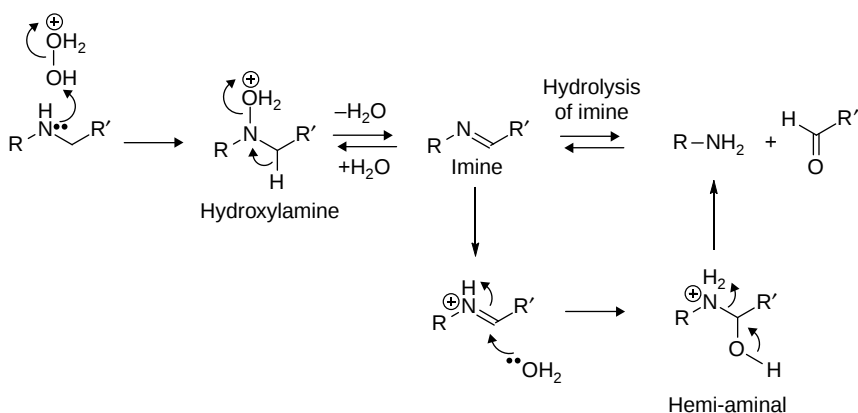


Figure 46 Proposed degradation mechanism of amine dealkylation.

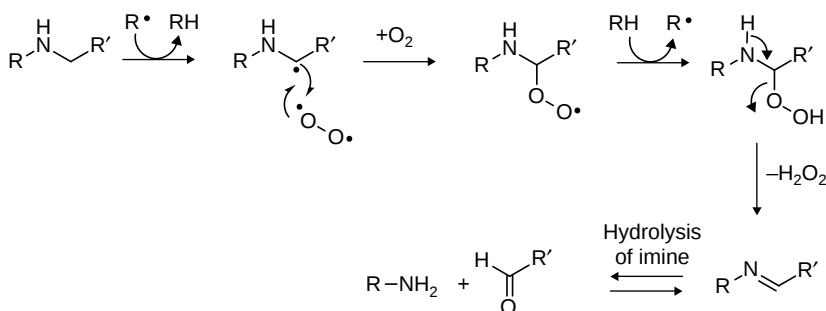


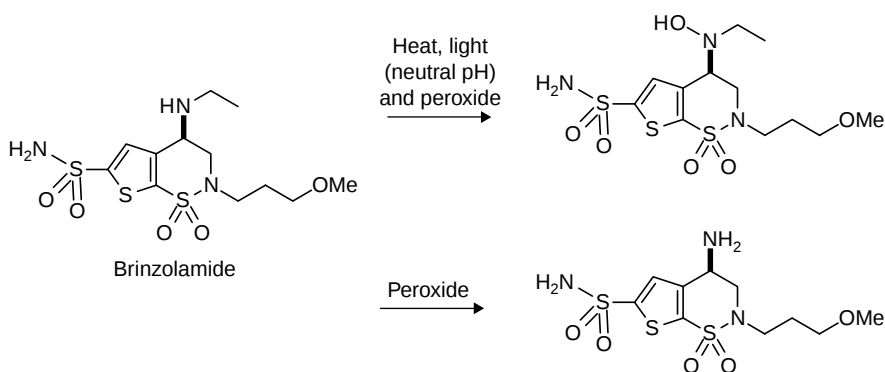
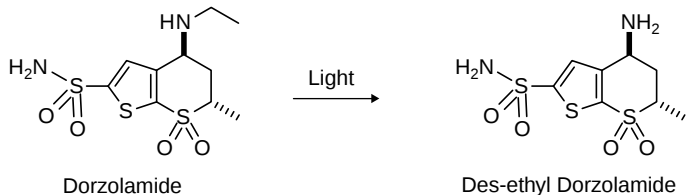
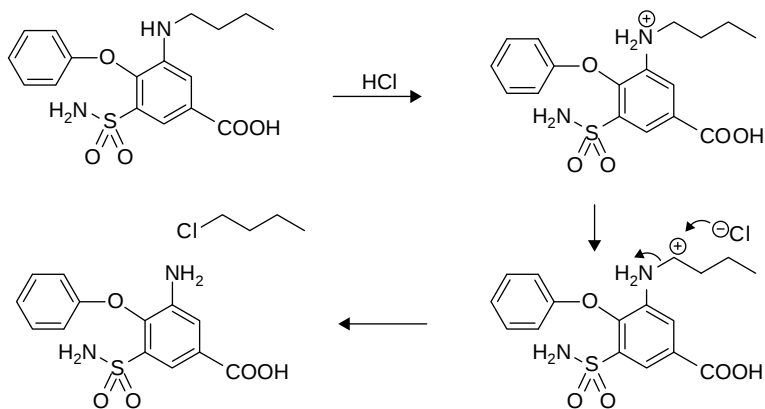
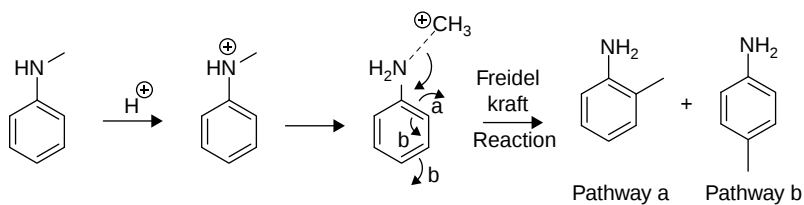
Figure 47 Radical-catalyzed oxidation of secondary amines.

The API bumetanide reacts under acidic conditions to convert the secondary amine into the corresponding primary amine (Fig. 50) (76). The mechanism is analogous to the Hofmann–Martius reaction resulting in the debutylated amine and *n*-butyl chloride.

Amines—Reaction with Formaldehyde and Other Aldehydes

Formaldehyde, along with other short-chain aldehydes such as acetaldehyde, is a low MW, volatile, reactive contaminant that can be present at low levels from a variety of sources (e.g., excipients such as polyethylene oxide, polyethylene glycol (PEG) (77,78) or from carbohydrate degradation (79), solvent contamination (59), packaging materials (60) etc.). Formaldehyde is known to react with amines (230) to form a reactive *N*-hydroxymethyl compound (a hemiaminal) that can further react with other nucleophiles. Reaction of formaldehyde with amino acids (80) can cause cross-linking of amino groups in gelatin to form an insoluble protein (81) which can inhibit dissolution of gelatin capsules (82). Because of its ubiquitous nature and propensity to react with pharmaceutical products (83) it has been suggested that exposure of APIs to formaldehyde should be considered for inclusion into routine “preformulation screens” (84).

The importance of and potential for reaction of APIs with aldehydes is illustrated by the L-tryptophan impurities incident. In 1989, a link between an outbreak of eosinophilia-myalgia syndrome (EMS) and the use of L-tryptophan as an over-the-counter dietary supplement was made. The outbreak of EMS resulted in more than 36 deaths and greater than 1500 serious illnesses (85). The L-tryptophan linked to this outbreak was manufactured by a single firm in Japan, and low levels of impurities present in lots manufactured by this firm have been

**Figure 48** Brinzolamide and dorzolamide dealkylation and oxidative degradation.**Figure 49** Dorzolamide photodegradation.**Bumetanide degradation****Hofmann-Martius reaction****Figure 50** Bumetanide degradation under acidic conditions.

postulated as the agents responsible for this toxic reaction. In particular, the impurity 1,1'-ethyldienebis(L-tryptophan), also known as EBT, was identified as a possible causal agent (86,87). This impurity is the reaction product of acetaldehyde and two molecules of L-tryptophan during the synthesis (Fig. 51). A definitive correlation of EMS with EBT or other impurities present in the L-tryptophan supplement was not made, however, possibly because of the difficulty of establishing an accurate animal model for the disease and the uncertainties in extrapolating such results to humans. Regardless, this significant tragedy has served to underscore the importance of developing an understanding of the potential impurities from either processing or degradation, and the toxicological implications.

Amines —The Maillard Reaction

The Maillard reaction, first described by Louis Maillard in 1912 (46), is not a single reaction but rather a collection of complex reactions that can occur between amines and reducing sugars, resulting in the production of brown pigments (88). This reaction is sometimes known as the "Maillard browning reaction," and has been extensively studied and reviewed (46,89,90,91,92). The article by Wirth et al. (49a) is an excellent starting point for understanding the chemistry of this degradation pathway and its relevance to pharmaceuticals. The Maillard reaction is classically represented by considering the reaction of a primary amine with a reducing sugar in its aldehydic form. The chemistry of the Maillard reaction is represented in Figure 52. The initial steps show that the anomeric carbon of a reducing sugar (e.g., lactose) is susceptible to nucleophilic attack by amines, which, upon loss of OH^- , gives rise to a reactive iminium ion. This iminium ion species can lose a proton and tautomerize to form an α -amino ketone that is known as the "Amadori rearrangement product" (ARP) (93).

A lesser-known degradation reaction of amines associated with the Maillard pathway is the propensity to undergo formylation upon extended storage in formulations with carbohydrates, especially reducing sugars such as lactose. This has been described by Wirth et al. in a study of the reaction of fluoxetine hydrochloride with lactose (49a) (Fig. 53).

The ARP is an important intermediate in the Maillard reaction. Under pharmaceutically relevant storage conditions the ARP can accumulate as an impurity to significant levels as was described in the cases of fluoxetine hydrochloride and pregabalin (94). As shown in Figure 53, in

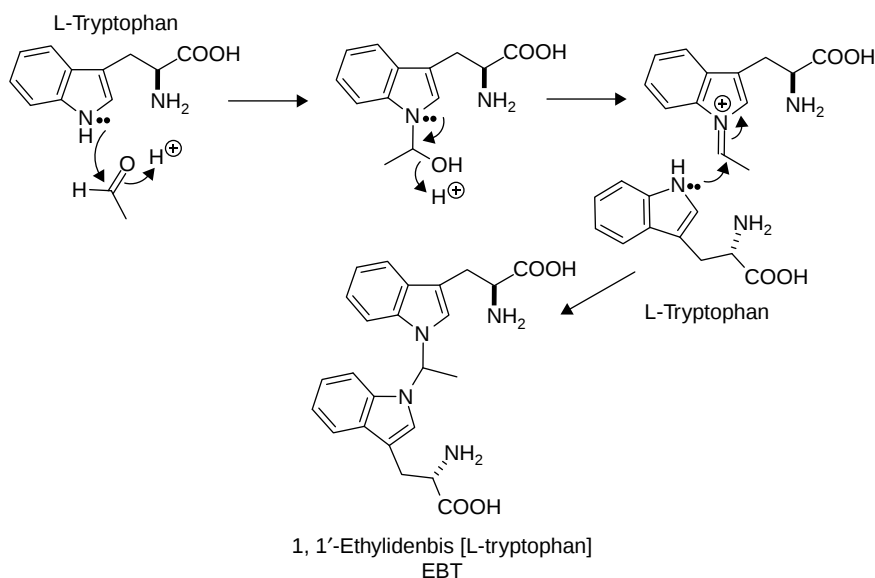


Figure 51 Reaction of L-tryptophan with acetaldehyde to form EBT.

the case of fluoxetine-lactose formulations the glycosylamine is also observed as a significant degradation product. It should be noted that although the structure of the fluoxetine-lactose ARP is shown as acyclic, it will exist in solution as a mixture of pyranose and furanose forms, both of which are diastereomeric (95). Thus, the ARP may be observed chromatographically as several peaks, possibly interconverting on-column. In addition to the glycosylamine and ARP

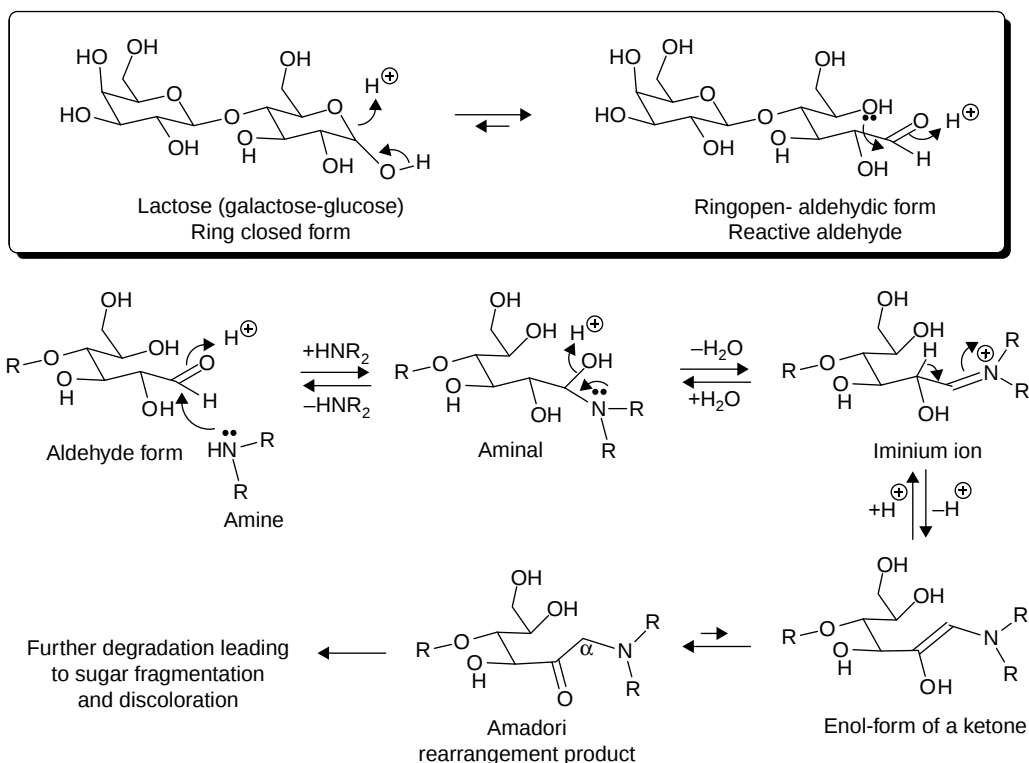


Figure 52 Initial steps of the Maillard browning reaction involving an Amadori rearrangement stable intermediate.

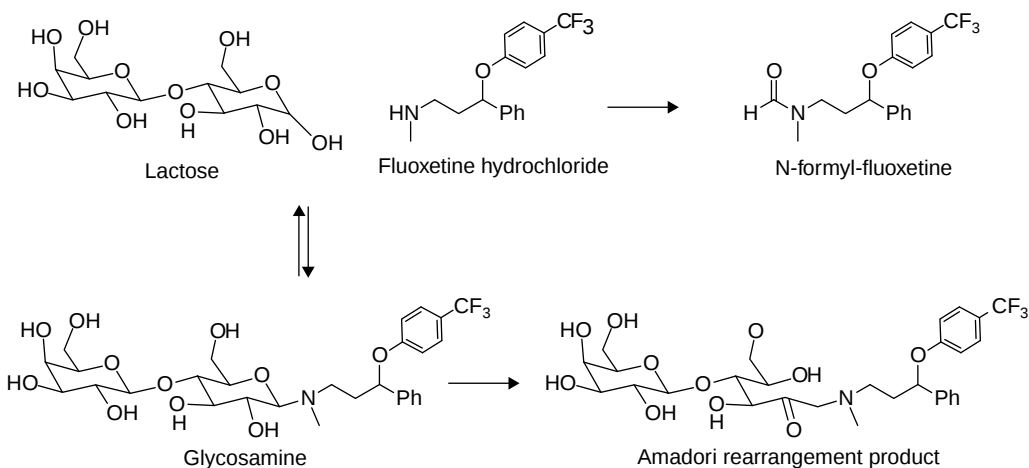


Figure 53 Maillard reaction of lactose and fluoxetine HCl yielding primary primary degradants glycosylamine, Amadori rearrangement product, and N-formyl fluoxetine.

degradation products, another significant product identified by Wirth et al. was *N*-formyl-fluoxetine. The degradation pathway leading to the *N*-formyl product was not clearly delineated, but must be the result of reaction of the secondary amine with a small fragment of the lactose skeleton. Glyoxal was proposed as a possible formylating agent that could result from the degradation of ARP. More recent studies have implicated formic acid as the formylating reagent, formed primarily from C1 of the reducing sugar via carbohydrate degradation (96). Regardless of the precise mechanism of formation, *N*-formylation should be considered as a possible degradation pathway for formulations of amine-containing APIs with reducing (e.g., lactose) and to a lesser extent, nonreducing (e.g., microcrystalline cellulose) carbohydrate excipients.

The Maillard reaction occurs much more readily with amorphous lactose than with crystalline lactose; therefore, this reaction is expected to be more of a concern for spray-dried material (97). Since the reaction is acid/base catalyzed, it is ideal to maintain a neutral pH environment with lactose-based products.

Amines: Reaction with Salts and Excipients Containing Carboxylic Acids

Amines are particularly prone to reaction with excipients and carboxylic acid counterions, as shown in Figure 54 for tartaric acid. Acids that are able to form cyclic anhydrides such as maleic acid, succinic acid, citric acid, and tartaric acid tend to undergo this reaction because the anhydride is relatively easy to form and reasonably reactive toward amines. The potential for a reaction with magnesium stearate or stearic acid can occur with 1° or 2° amines, but is particularly of concern when the API contains a primary amine. In the case of norfloxacin, formation of a stearyl amide derivative was observed in tablets containing magnesium stearate after prolonged storage at 60°C (Fig. 55) (98). Since magnesium stearate can be derived from multiple sources, the presence of other fatty acids (FAs) [e.g., palmitic (C16), arachidic (C20), and behenic (C22) acids] can lead to more than one FA amide derivative.

The potential for reaction of a primary amine salt with its counterion is exemplified by seproxetine maleate (99). In formulations with pregelatinized starch, after storage at 25 °C and 40°C for 3 months, a 1,4-Michael addition adduct was formed between the primary amine of

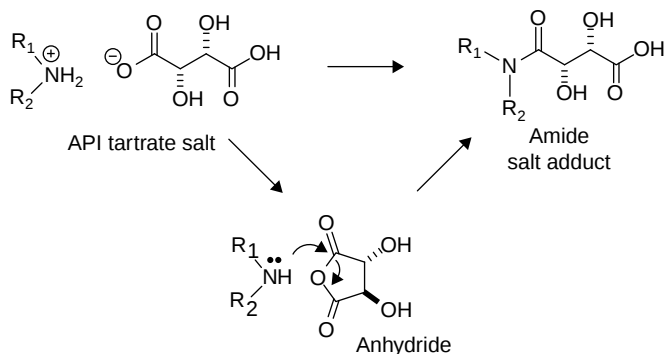


Figure 54 Amine reaction with tartaric acid.

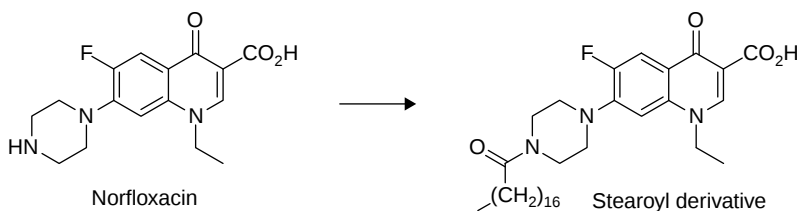


Figure 55 Norfloxacin reaction with magnesium stearate.

1,4-Michael addition

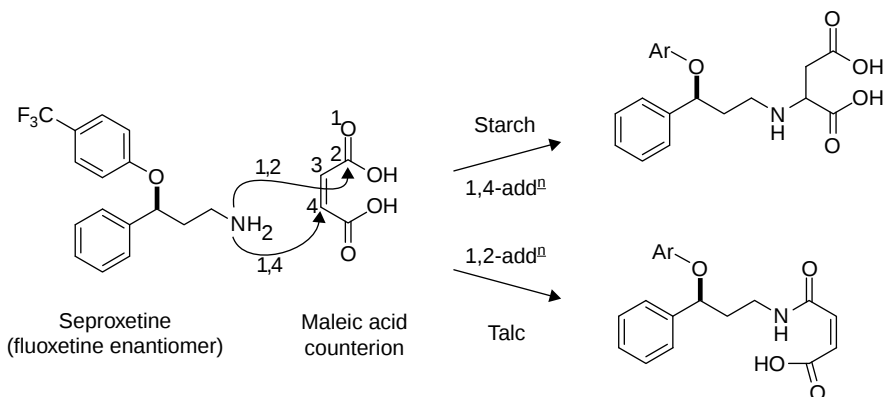
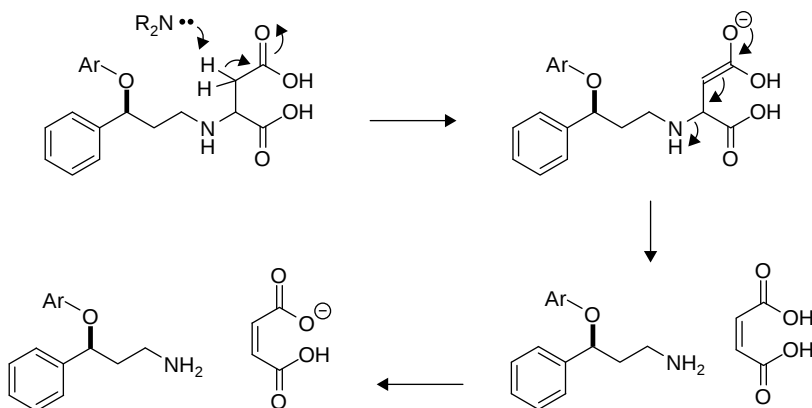
 β -Elimination

Figure 56 Reaction of a primary amine with its maleate counterion in stressed formulations.

seproxetine and maleate (Fig. 56). In formulations containing talc, after similar storage, an amide linkage was formed (1,2-addition adduct). While these reactions were not specifically excipient-induced (i.e., the same reactions can occur without the presence of excipients), it is still of interest from a formulation viewpoint. It is noteworthy that these reactions were greatly suppressed when using the fumarate salt (the *trans* double bond configuration of maleate). The 1,4-Michael addition reaction is a reversible reaction. The amine in the 4-position is a good leaving group and the presence of a base could enable a base catalyzed β -elimination to occur. The formation of these adducts could be considered to be very pH sensitive and adjustment of the pH may afford significant changes in the formation of these degradants.

Amines: Reaction with Formulation Components

Amine containing APIs have also been known to react with other formulation components such as flavoring agents and even enteric coating constituents. An example of the reaction of a primary amine with a flavoring agent is illustrated in Figure 57 (100). In this example the API was formulated as a ready-to-use liquid, oil-based formulation, and vanillin was one of the flavoring components. Upon long-term storage, low levels of new impurities were observed, several of which appeared to be unstable and difficult to isolate. It was determined that the degradation-related

impurities were the result of the reaction of the primary amine with the aldehydic functionality of vanillin, leading to *cis/trans*-imines, and also to inversion of the chiral center.

Another example of an amine reacting with a formulation component is found in the case of duloxetine hydrochloride (101). This example, which is also discussed in chapter 2 is summarized in Figures 58 and 59. In this example, the secondary amine of duloxetine hydrochloride reacted with the enteric coating polymer hydroxypropyl methylcellulose acetate succinate (HPMCAS) to form a succinamide degradation product. This reaction occurred under both stress conditions (60°C for 14 days) and during formal stability studies (30°C/60% relative humidity and 40°C/75% relative humidity). The reaction is especially interesting in that there was a physical separation (different physical layers) of the API from the HPMCAS enteric polymer. Since it was concluded from spiking experiments that duloxetine hydrochloride did not react with free succinic acid present in the enteric polymer layer, an alternate pathway was proposed. It was postulated that the degradation product was forming either via migration of duloxetine hydrochloride to facilitate intimate contact with the polymer, or by degradation of

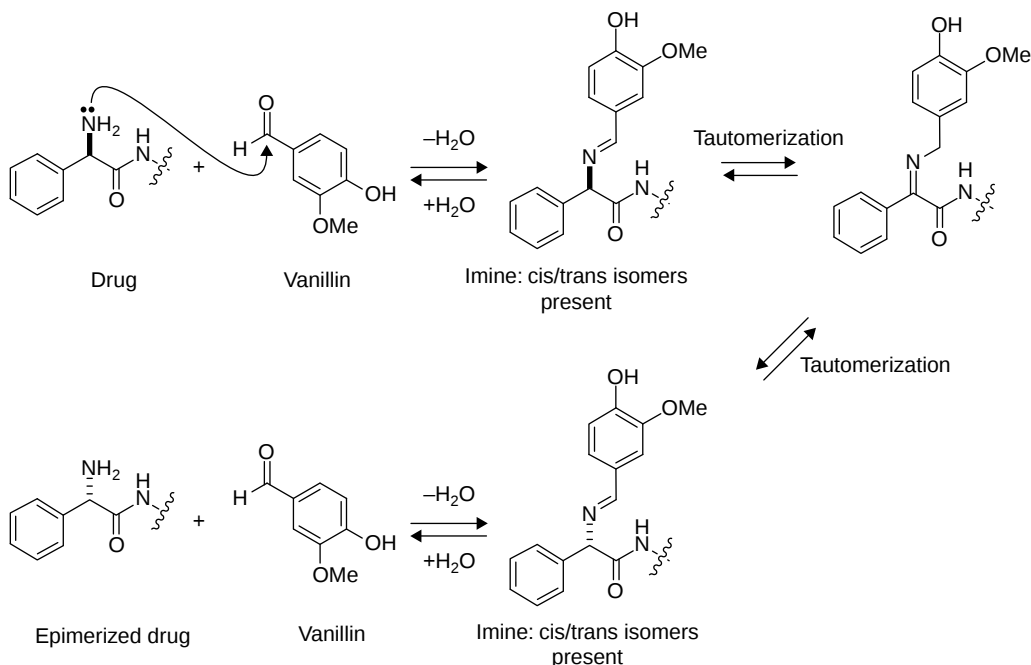


Figure 57 Aldehydes plus amines: reaction of API with flavoring agent.

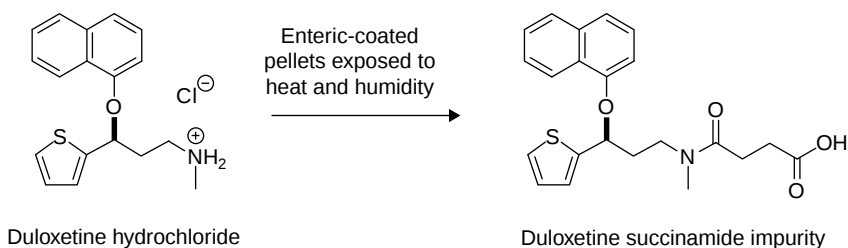


Figure 58 Structures of duloxetine hydrochloride and a low-level impurity formed upon aging of enteric-coated pellets.

the polymer to form succinic anhydride, which could migrate to the API layer of the formulation (Fig. 59). The degradation reaction was minimized by increasing the thickness of the barrier layer between the API and enteric polymer layers.

In yet another example of an amine reacting with a formulation component is that of meropenem (102). Meropenem is a secondary amine containing drug substance that is formulated as a blend of crystalline drug and sodium carbonate. It was determined that meropenem exists partially as a covalent, carbon dioxide adduct (a carbamic acid salt) in both the solid powder and in the reconstituted solution for injection. Under acidic conditions, the carbamate protonates and the resulting carbamic acid derivative rapidly loses carbon dioxide to regenerate the parent drug. The overall degradation reaction is represented in Figure 60.

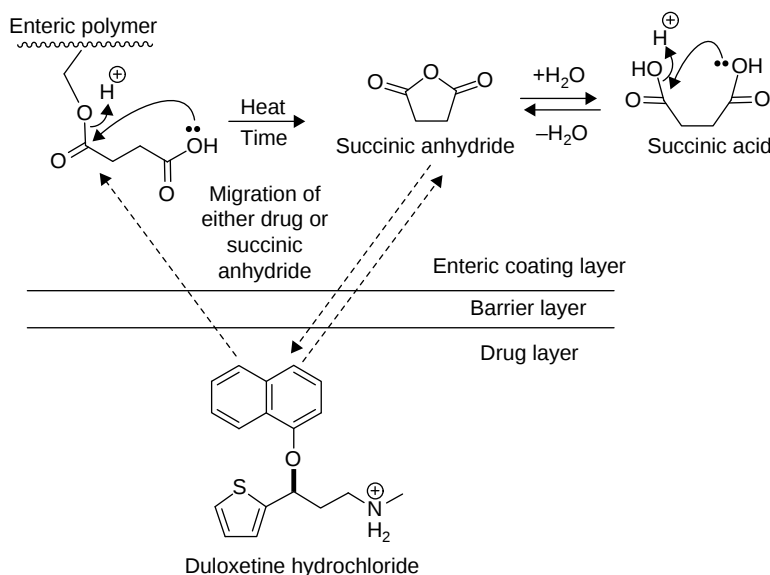


Figure 59 Proposed pathways for the interaction of duloxetine hydrochloride with HPMCAS to form duloxetine succinamide.

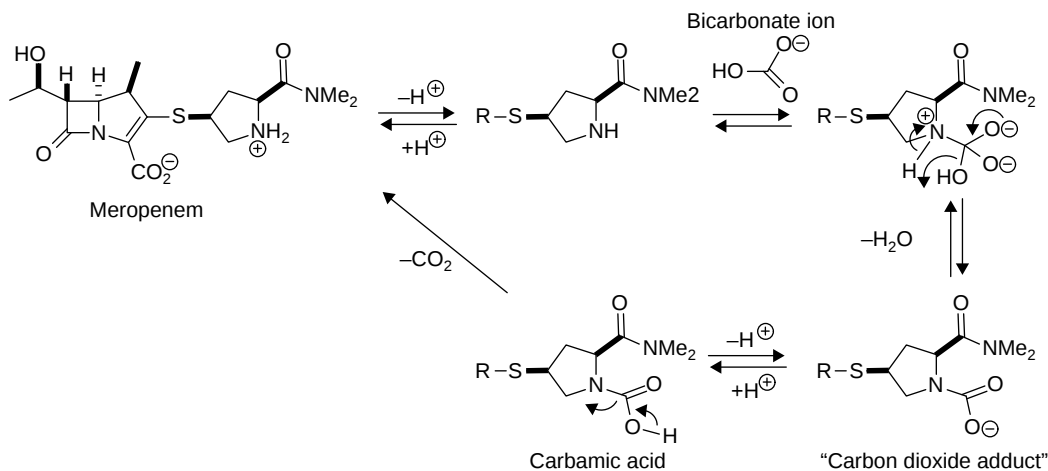


Figure 60 Meropenem, a secondary amine, reacts with sodium carbonate to form a carbon dioxide adduct.

Imines

Imines readily hydrolyze in the presence of water, especially under basic or acidic conditions. Figure 61 shows the acid-catalyzed hydrolysis of imines. When developing methods to detect imines, it is often necessary to use neutral pH conditions to optimize the stability of the imine. An example of imine formation and hydrolysis is found in the degradation chemistry of the API sertraline hydrochloride (Fig. 62) (103).

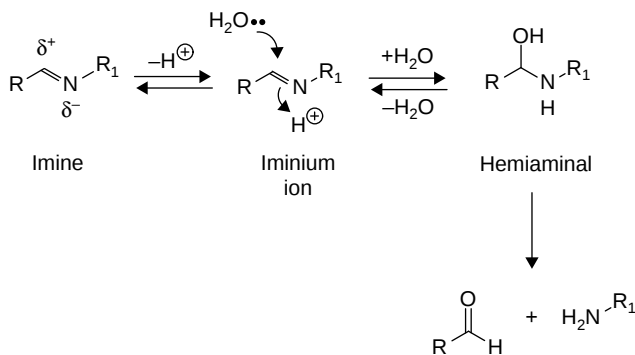


Figure 61 Imine hydrolysis.

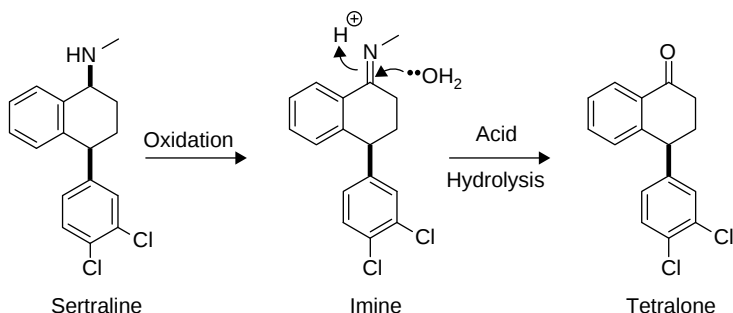


Figure 62 Sertraline hydrochloride imine formation and hydrolysis under acidic conditions.

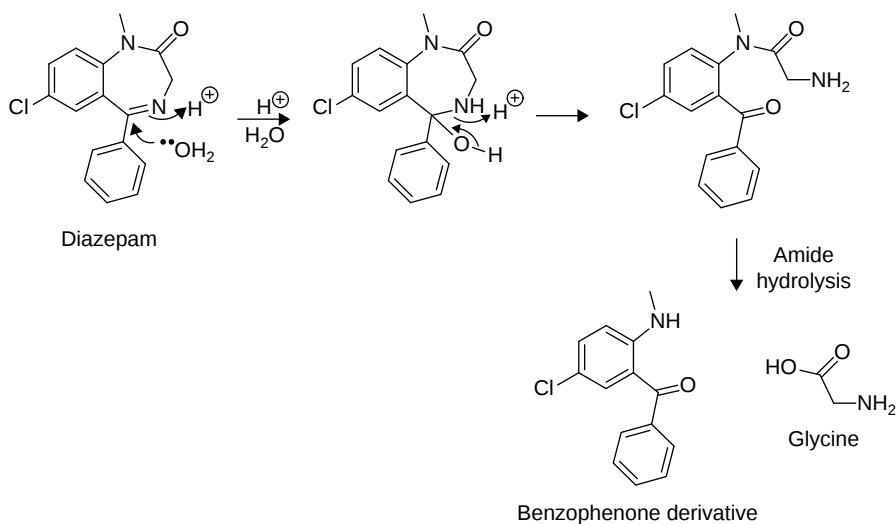


Figure 63 Diazepam acid hydrolysis.

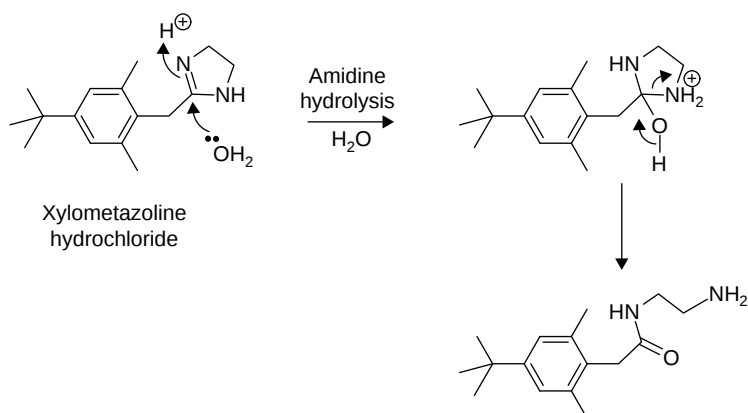


Figure 64 Xylometazoline ring opening hydrolysis.

Other API examples include methaqualone (104) and terbutaline sulfate in which the amine oxidizes to an imine followed by imine hydrolysis (105). In the case of diazepam under acid hydrolysis conditions, the imine undergoes decomposition to the corresponding benzophenone (Fig. 63) (106).

In the case of xylometazoline hydrochloride, imine hydrolysis chemistry occurs to open the 4,5-dihydro-1H-imidazole ring (Fig. 64) (107). Similar degradation chemistry is observed for the following APIs: flurazepam hydrochloride (108), clorazepate dipotassium (109), clonazepam (110), methaqualone (111), chlordiazepoxide, and oxazepam (23).

Hydrazines

Hydrazines $[(R)_2-N-N-(R)_2]$ are susceptible to hydrolysis similar to amines, and are susceptible to oxidation. Procarbazine hydrochloride, which contains a hydrazine moiety, undergoes oxidation

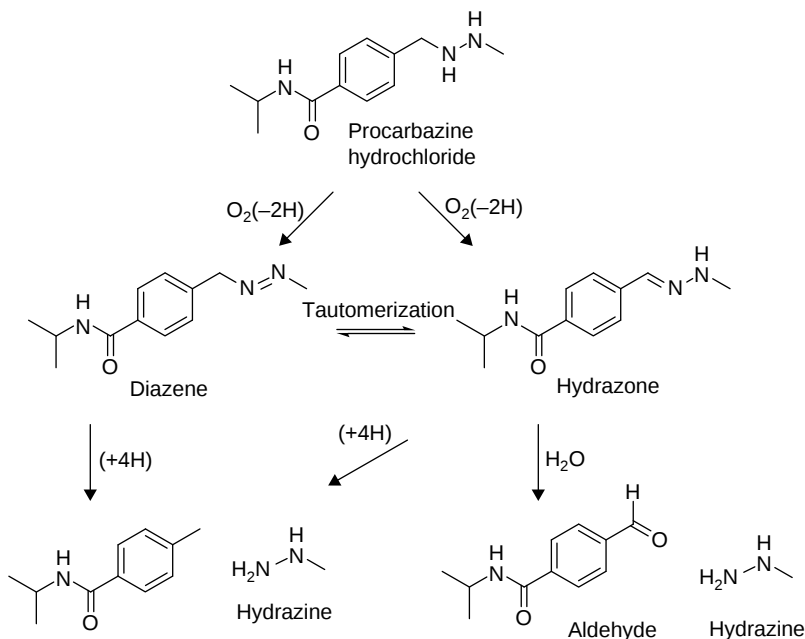
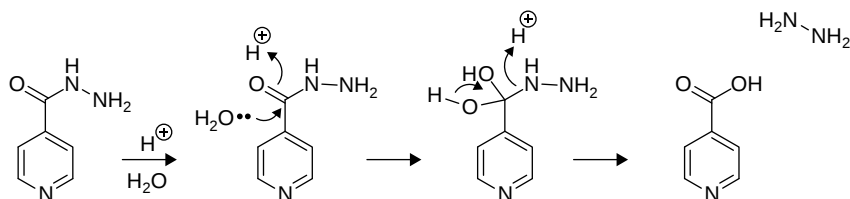
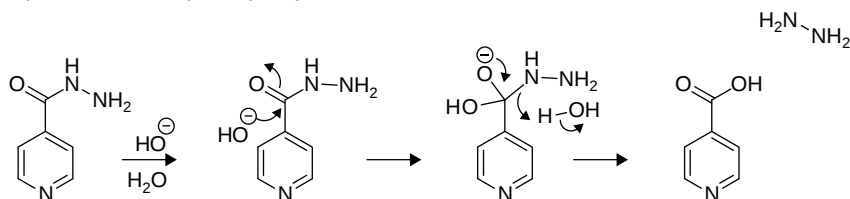


Figure 65 Degradation of procarbazine hydrochloride.

Proposed acid catalyzed hydrolysis mechanism



Proposed base catalyzed hydrolysis mechanism

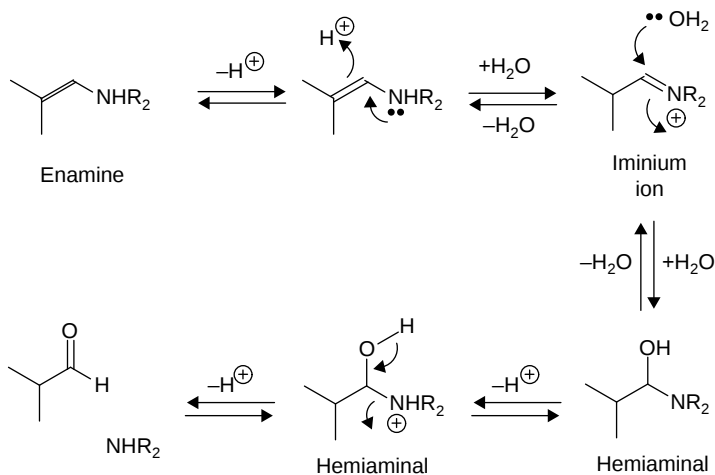
**Figure 66** Hydrolysis of acyl-hydrazines illustrated by the hydrolysis of isoniazid.

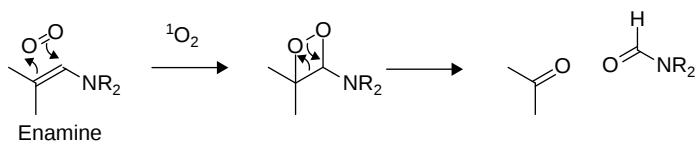
by atmospheric oxygen in the presence of moisture or in aqueous solution to form the diazene and hydrazone compounds (which are tautomeric forms) shown in Figure 65. The hydrazone undergoes further decomposition in the presence of molecular oxygen and water to form the resulting aldehyde and hydrazine (analogous to imine degradation chemistry) (112). Hydrazines are genotoxic alerting structures (70) and may present potential genotoxic impurity concerns.

Acyl-hydrazine compounds $[(RC(=O)-N-N-(R)_2)]$ undergo hydrolysis reactions similar to amides and lactams (Fig. 11) (Fig. 66 for an example of acyl-hydrazine hydrolysis in the case of isoniazid) (113).

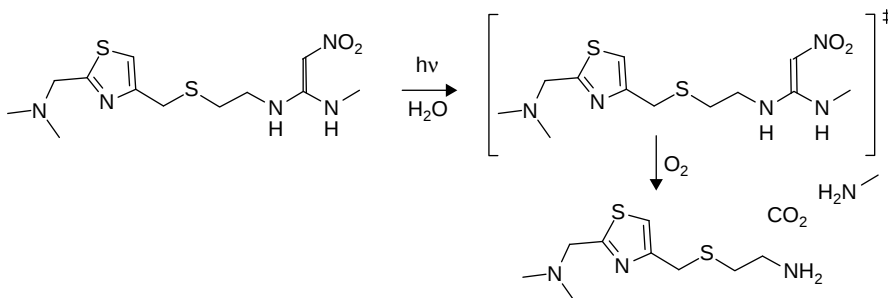
Enamines

Acid-catalyzed hydrolysis of enamines (last step of the Stork enamine reaction) (114) involves conversion to an iminium ion which undergoes hydrolysis to the ketone as shown in Figure 67.

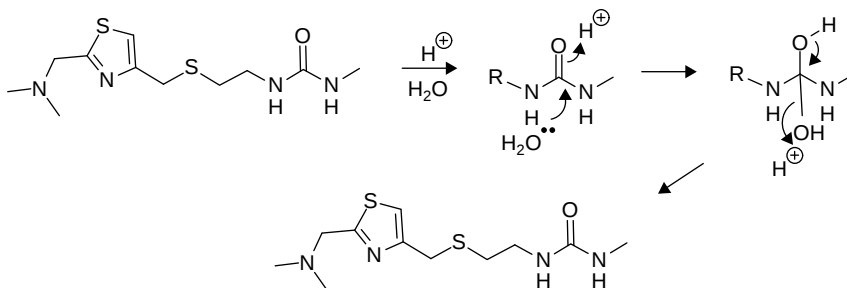
**Figure 67** Mechanism of enamine hydrolysis.

**Figure 68** Enamine reaction with singlet oxygen (photochemistry).

Nizatidine photolysis



Nizatidine hydrolysis (HCl) of photodegradant

**Figure 69** Nizatidine degradation chemistry.

The iminium ion undergoes hydrolysis quite readily since there is a contributing resonance form with a positive charge on the carbon (115).

Enamines have an electron rich double bond that is susceptible to reactivity with singlet oxygen (formed through the photosensitization of ground state molecular oxygen) as shown in Figure 68.

The API nizatidine contains enamine functionality (Fig. 69). Degradation of the enamine functionality under acid and basic conditions yields the subsequent amine. After irradiation with a mercury lamp in an aqueous solution, nizatidine degraded to the corresponding urea compound from the addition of water to the nitro-substituted enamine, followed by cleavage (consistent with the enamine reacting with singlet oxygen, Fig. 68 (116)).

Nitro Groups

Nitroaromatic groups are susceptible to photochemical reactivity (52). A well-known nitro-group degradation reaction occurs for the API nifedipine (Fig. 70). Under ultraviolet as well as visible radiation, the nitro group of nifedipine is rapidly converted to a nitroso compound

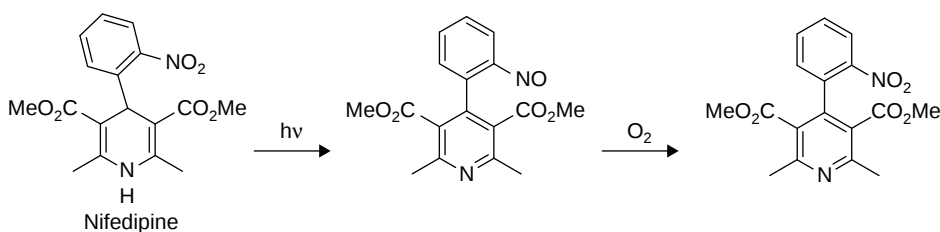


Figure 70 Nifedipine photochemistry.

along with aromatization to a substituted pyridine ring (117). In the presence of molecular oxygen, the nitroso functionality is re-oxidized to the nitro derivative (118). See chapter 7, Figure 9, for the case of metronidazole, illustrating a rearrangement often observed with nitrated five-membered heterocycles.

Sulfonyl Chemistry

Sulfonamides

Sulfonamides are generally susceptible to acid hydrolysis, but are not readily hydrolyzed under basic conditions (Fig. 71) (119). Primary alcohols react rapidly only with *N,N'*-disubstituted sulfonamides to yield sulfonic esters. Sulfonamides are not susceptible to oxidation since the sulfur is already fully oxidized. The formation of sulfonic acid esters may be a potential genotoxic degradant problem (120). These esters have the tendency to be alkylating agents because of the ability of the sulfonic acid to act as a very good leaving group. Nucleophilic (S_N2') attack on the R' can occur.

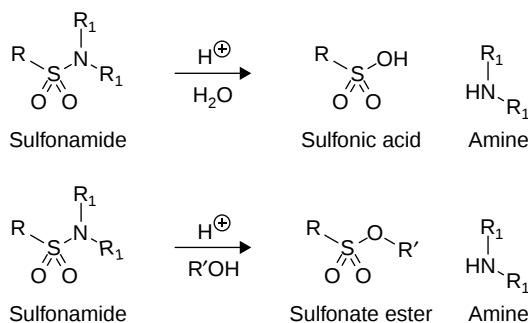


Figure 71 Sulfonamide degradation chemistry.

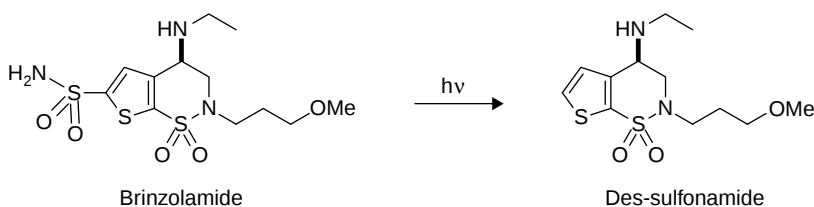


Figure 72 Photodegradation chemistry of brinzolamide.

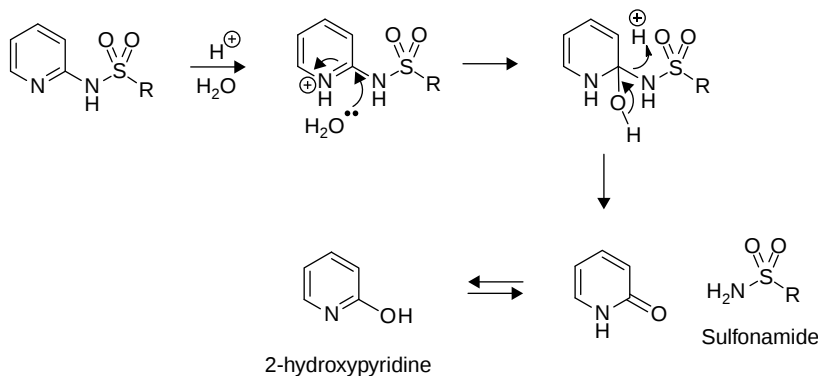
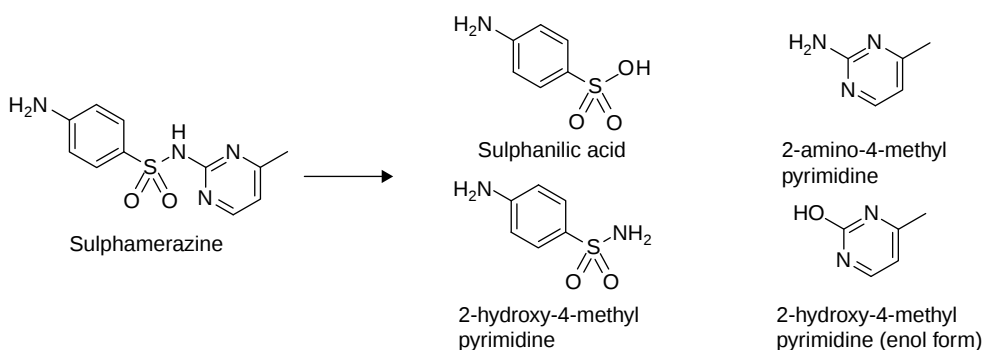


Figure 73 Hydrolysis of pyridine-2-aminosulfonamide.



Proposed hydrolysis mechanisms

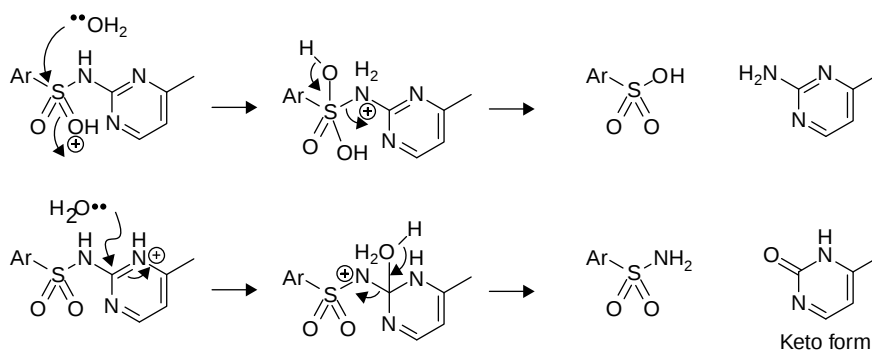


Figure 74 Sulphamerazine degradation chemistry.

Photolysis at 254 nm of arylsulfonamides of aliphatic amines yields the corresponding free amine (121). In the presence of light, the API brinzolamide in solution undergoes cleavage of the sulfonamide to yield the corresponding des-sulfonamide (Fig. 72) (122).

The API sulphamerazine undergoes hydrolysis on the sulfonamide group to form the products shown in (Fig. 74) (123). Additionally, aryl hydrolysis also occurs to yield the 2-hydroxy-4-methylpyrimidine and sulphanilamide. The API sulfamethazine degrades under acidic conditions to produce sulfanilic acid and 2-amino-4,6-dimethyl-pyrimidine (124). The hydrolysis of the 2-aminosulfonamide-pyridine (or substitute pyrimidine or quinoline for

pyridine) systems to the aminosulfonamide and 2-hydroxypyridine/pyrimidine/quinoline is a lesser known but common occurrence in the hydrolytic degradation of APIs with this functionality (Fig. 73). This reaction occurs faster under basic conditions than acidic.

Sulfonylureas

Sulfonylureas undergo hydrolysis as shown in the mechanistic scheme in (Fig. 75) (125). Under acid-catalyzed conditions, water addition occurs followed by loss of an amine and carbon dioxide to yield the corresponding sulfonamide. It was proposed that initial protonation is the rate-determining step in the hydrolysis.

An example of an API containing the sulfonylurea functionality is glibenclamide (Fig. 76) (122). Acid degradation produces the corresponding sulfonamide, amine, and carbon dioxide.

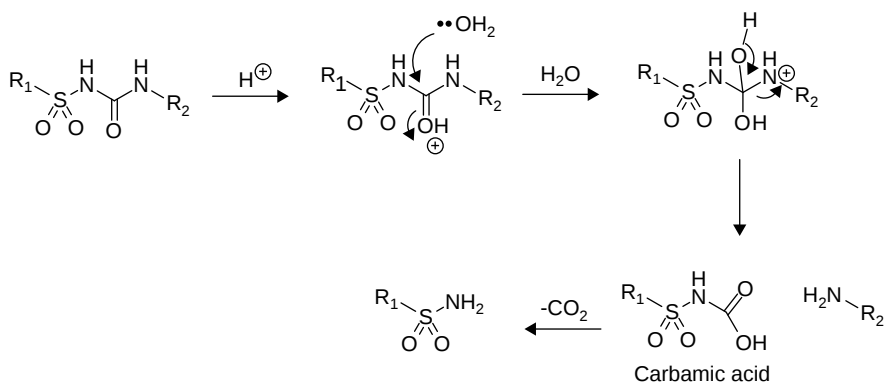


Figure 75 Sulfonylurea hydrolysis chemistry.

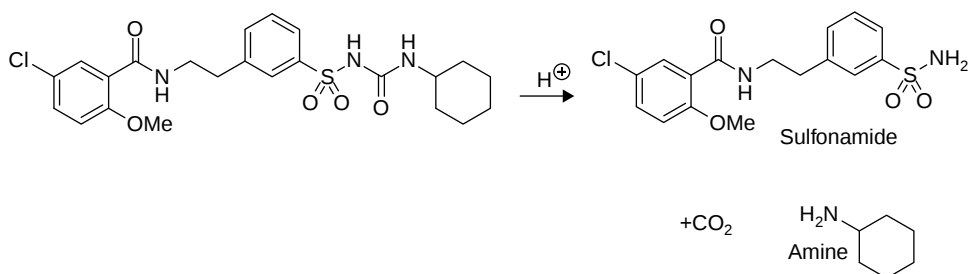


Figure 76 Glibenclamide sulfonylurea hydrolysis chemistry.

Thiols

Similar to hydroxyls and alkyl halides, thiols can hydrolyze to the corresponding hydroxyl via acid or base catalysis, releasing hydrogen sulfide in the process (Fig. 77). Thiols are ionizable and will typically exist as the anion at pHs higher than 8–9 (pK_a 's depend, of course, on the substituents and can vary substantially).

Thiols are susceptible to oxidation by peroxides, molecular oxygen, and other oxidizing processes (e.g., radical-catalyzed oxidation) (Fig. 78). Because thiols easily complex with transition metals it is believed that most thiol autoxidation reactions are metal-catalyzed (126).

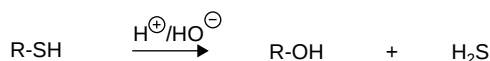


Figure 77 Thiol hydrolysis reaction scheme.

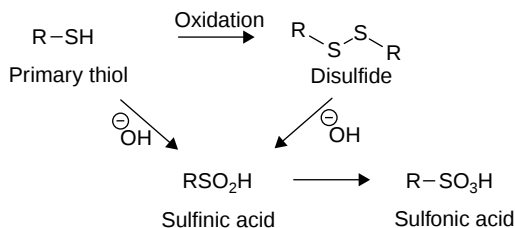


Figure 78 Oxidation of thiols.

Autoxidation of thiols is enhanced by deprotonation of the thiol to the thiolate anion. Thiol oxidation commonly leads to disulfides, although further autoxidation to the sulfinic and, ultimately, sulfonic acid can be accomplished under basic conditions. Disulfides can be reduced back to the thiol (e.g., upon addition of a reducing agent such as dithiothreitol). Thiols are nucleophilic and will readily react with available electrophilic sites. For a more thorough discussion see Hovorka and Schöneich (126) and Luo et al. (127).

Ethers, Thioethers

Both ethers and thioethers can be hydrolyzed via acid-catalysis to the corresponding alcohol or thiol, respectively, but are reasonably stable to neutral and basic conditions (Fig. 79).

Ether hydrolysis is observed for the API timolol maleate. Under pH 5 aqueous autoclave conditions (120°C), three degradants were isolated. Figure 80 depicts the degradants and the proposed degradation pathway involving (i) rearrangement to isotimolol, (ii) ether hydrolysis to form 4-hydroxy-3-morpholino-1,2,5-thiadiazole, and (iii) oxidation of the sulfur to form 4-hydroxy-3-morpholino-1,2,5-thiadiazole-1-oxide (128).

Duloxetine hydrochloride is an example of an aryl ether that is particularly unstable to hydrolysis under acidic conditions (see also chap. 2, Fig. 12 and 13) (100). The acid instability led to the development of an enteric-coated formulation to protect the compound from the acidic environment of the stomach. The reason for the susceptibility to hydrolysis is the stability of the cationic intermediate (Fig. 81), which is stabilized by delocalization into the aromatic thiophene ring.

Thioether hydrolysis is observed for the API cefamandole nafate under slightly acidic, slightly basic and aqueous photolytic conditions, leading to the thiol-substituted tetrazole and alcohol (Fig. 82) (129).

Additional examples of thioether hydrolysis in solution include the API moxalactam disodium to form thiotetrazole (130). Thioethers are susceptible to oxidation (both peroxide and radical mediated) to sulfoxides and sulfones (Fig. 83). It is worth noting here that sulfoxides are

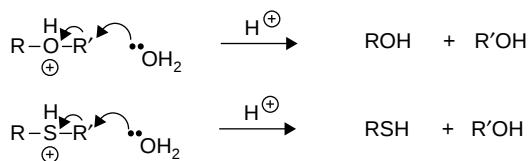
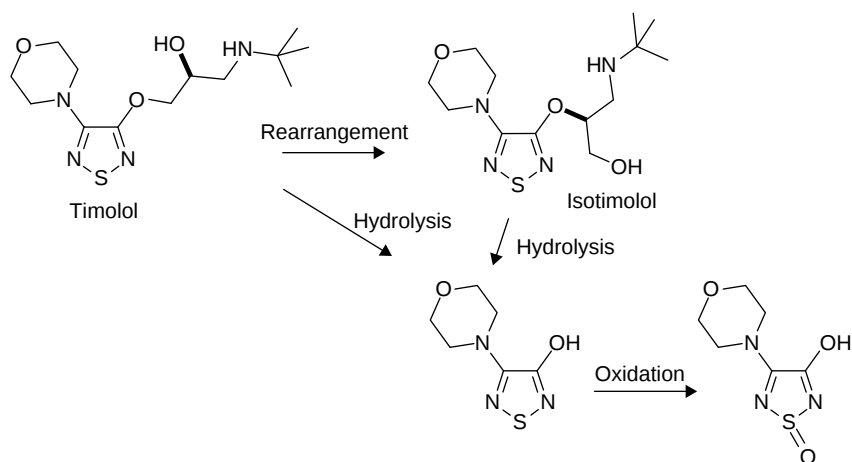


Figure 79 Ether, thioether hydrolysis under acidic conditions.



Proposed rearrangement mechanism

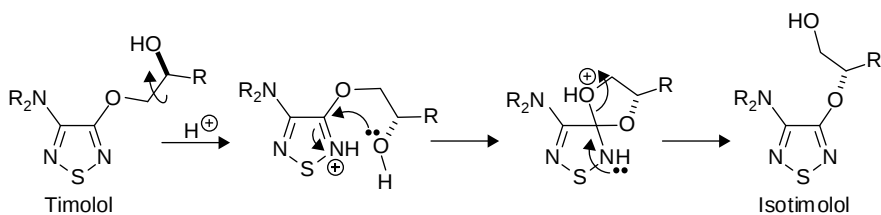


Figure 80 Proposed aqueous solution degradation pathway for timolol maleate.

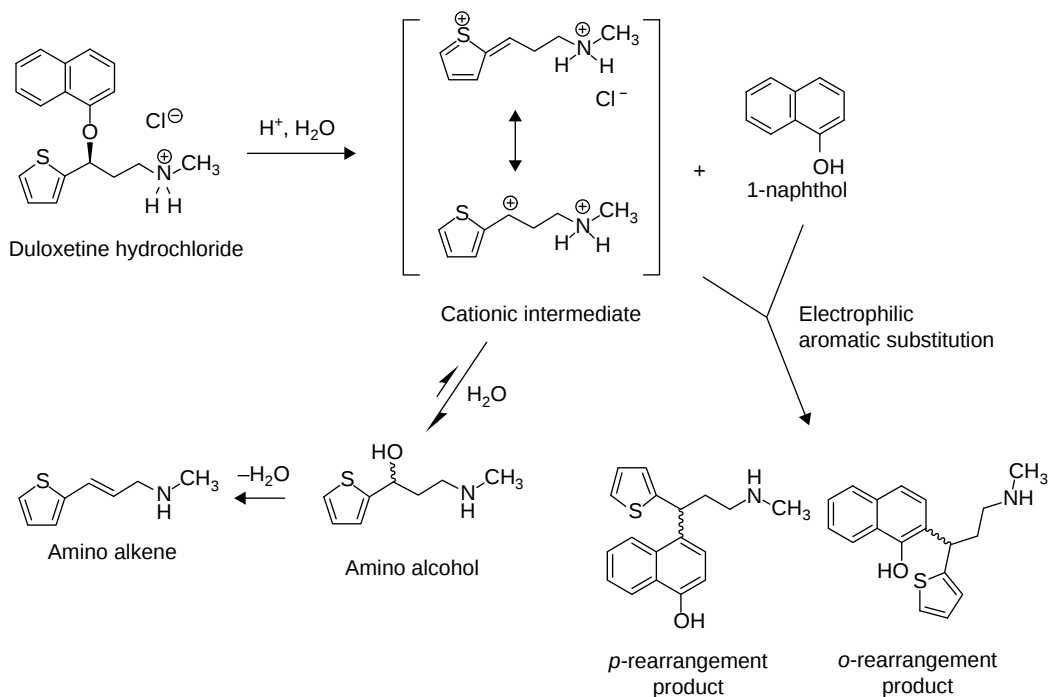
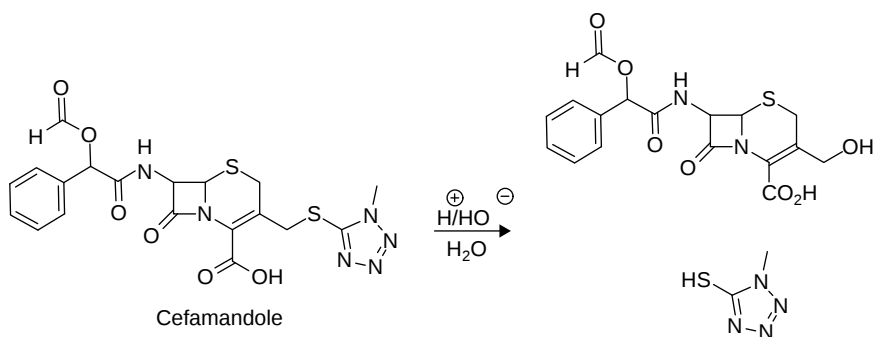
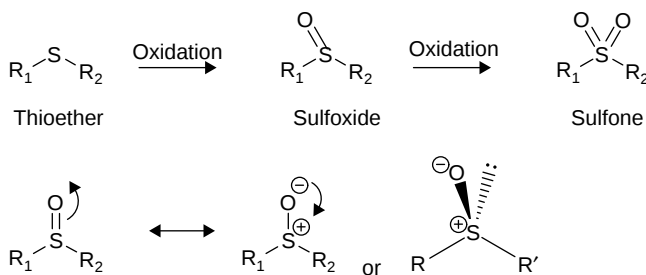
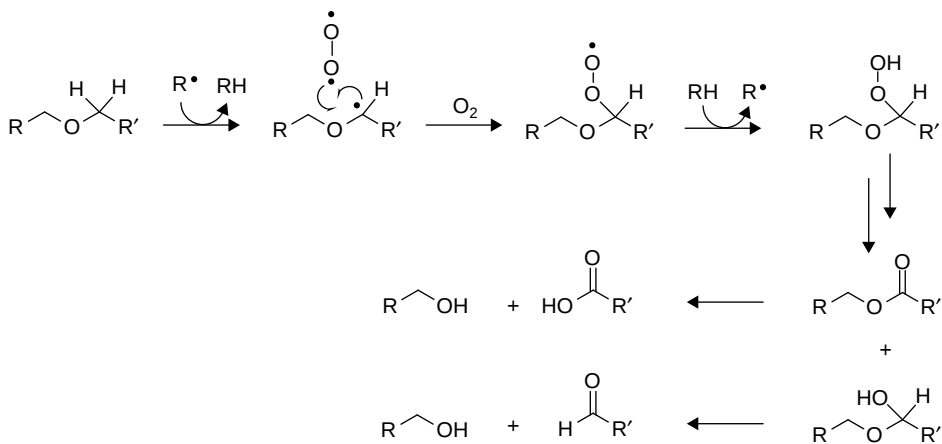


Figure 81 Hydrolysis of the ether linkage of duloxetine hydrochloride under acidic conditions.

**Figure 82** Cefamandole thioether hydrolysis.**Figure 83** (Upper) Oxidation of thioethers. (Lower) Different representations of a sulfoxide, including the chiral representation.**Figure 84** Oxidative degradation of ethers.

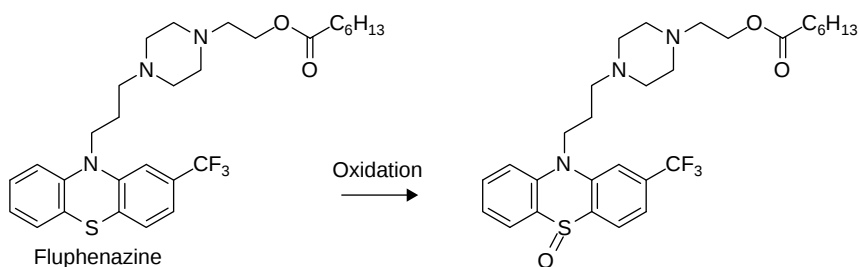


Figure 85 Fluphenazine enanthate oxidation to sulfoxide.

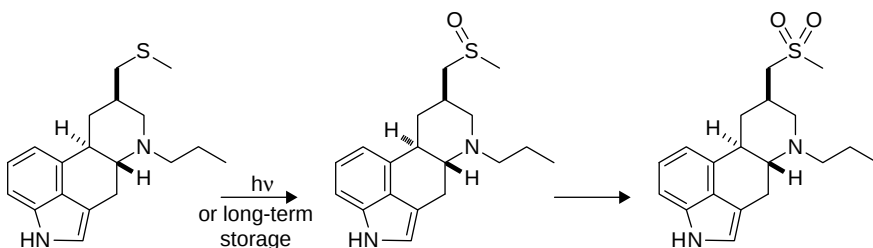
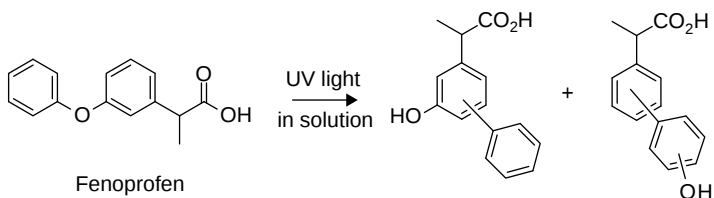


Figure 86 Pergolide mesylate oxidation to sulfoxide and sulfone.



Proposed mechanism

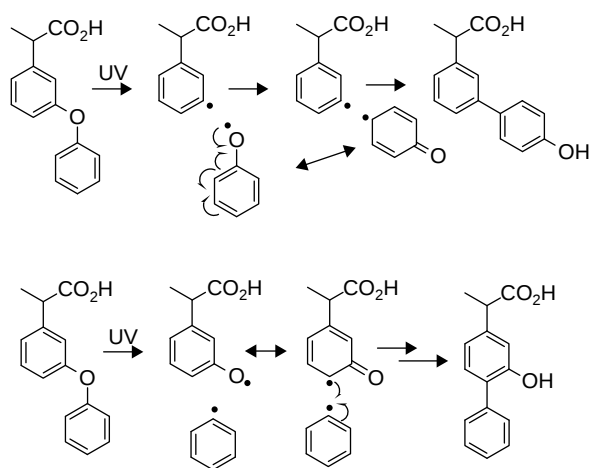


Figure 87 Fenopropfen calcium photodegradation and proposed mechanism.

chiral, and the formation of a sulfoxide from a thioether introduces a new chiral center into the molecule.

Ethers are susceptible to autoxidation (i.e., radical-initiated oxidation) to form unstable hydroperoxides. These hydroperoxides can decompose through various pathways to yield the corresponding alcohols, carboxylic acids, and aldehydes (Fig. 84). Ether oxidation can occur at the carbon α to the oxygen. Initiation occurs to generate a radical stabilized by the α -oxygen. Molecular oxygen can then add (at the diffusion controlled rate in solution), followed by hydrogen atom abstraction to yield the corresponding hydroperoxide which can subsequently decompose to the ester and aldehyde secondary degradation products (Fig. 84). Alternatively, the hydroperoxy radical may decompose prior to the formation of the corresponding hydroperoxy compound, resulting in a similar profile of products.

The API fluphenazine enanthate undergoes oxidation of a secondary aryl thioether to the resulting sulfoxide (Fig. 85) (131).

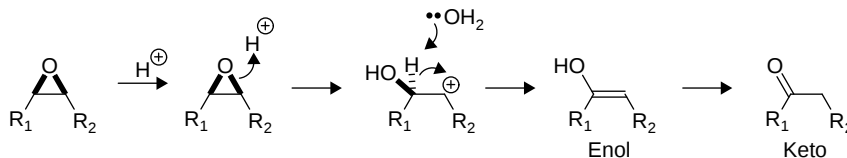
Additional API examples of thioether to sulfoxide degradation include cimetidine (132), timolol (133), nizatidine (134). The API pergolide mesylate degrades to the corresponding sulfoxide as well as the sulfone (Fig. 86) under visible light exposure of 3×10^6 lux hours or upon aging under long-term storage conditions (135).

The API fenoprofen calcium is diaryl ether. Degradation of fenoprofen under intense ultraviolet light in solution yields a mixture of isomeric biphenyls via a photo-Fries rearrangement mechanism (Fig. 87) (136).

Epoxides

Epoxides are typically very reactive functional groups that are susceptible to nucleophilic attack by water (hydrolysis to form diols) or other nucleophiles. The three-membered oxirane ring contains significant strain and the ring opening relieves this strain. Hydrolysis to the diol is catalyzed by both acid and base. The diol formed from hydrolysis of the epoxide ring may react further by dehydration and tautomerization to form a ketone, as shown in Figure 88.

Epoxide ring opening S_N1



Epoxide ring opening S_N2'

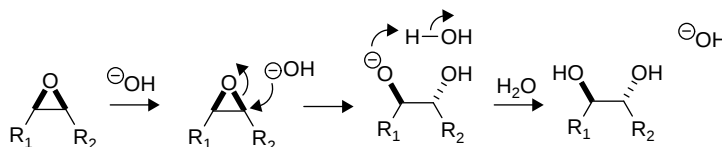
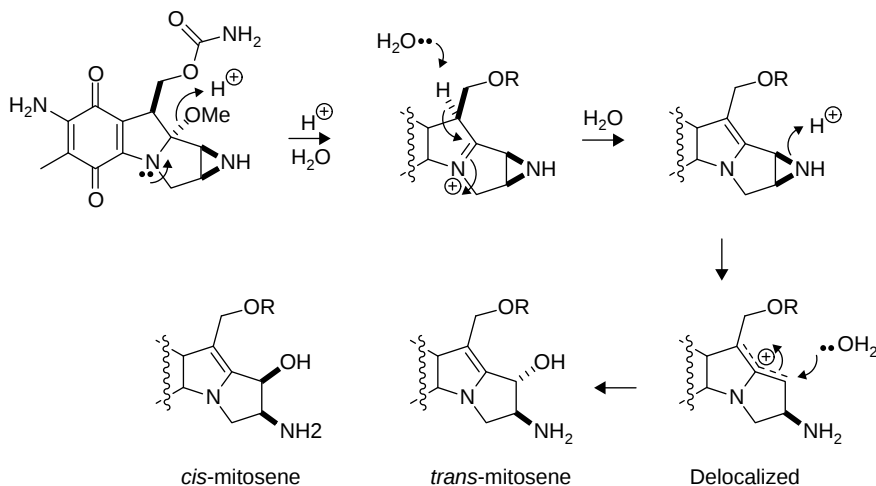


Figure 88 Hydrolysis of an epoxide.

Aziridines

Aziridines, nitrogen containing three-membered rings, are also subject to ring opening reactions due to the high ring strain present in the system. The API mitomycin C contains an aziridine ring. In aqueous acidic solutions, aziridine ring opening occurs to form a planar allylic cation. Addition of water at to the planar allylic cation at C1 can occur from both faces

Mitomycin C acid-catalyzed aziridine hydrolysis



Mitomycin C base-catalyzed hydrolysis

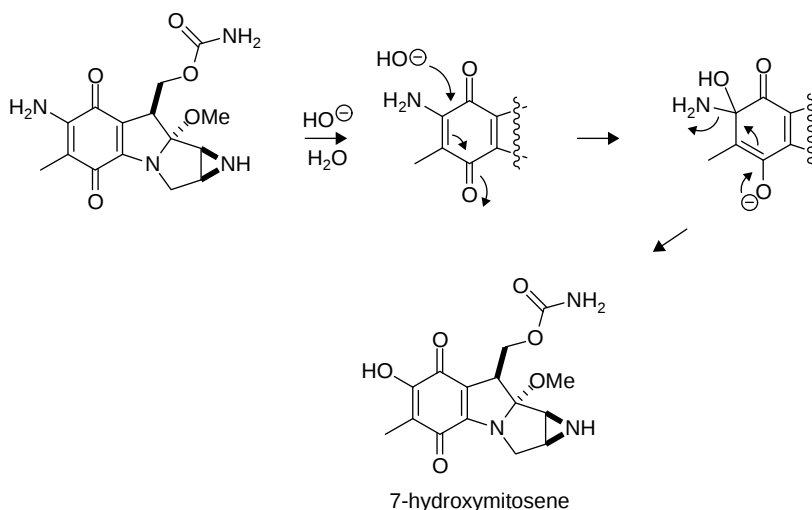


Figure 89 Hydrolysis reactions of Mitomycin C involving (*upper*) aziridine ring opening to *cis*- and *trans*-mitosene, and (*lower*) base-catalyzed hydrolysis of the 7-amino group.

(from above or from below) yielding *cis*- or *trans*-mitosene (Fig. 89) (137). In basic solutions, nucleophilic attack occurs to yield 7-hydroxymitosene.

Hydroxyl Groups

Hydroxyl groups can act as nucleophiles, although they are less nucleophilic than amines or thiols. Under acidic conditions, hydroxyls can be eliminated in a dehydration reaction (Fig. 90). Elimination reactions can occur as an E1 reaction (elimination unimolecular) or E2 reaction (elimination bimolecular). The E1 elimination mechanism proceeds through formation of a

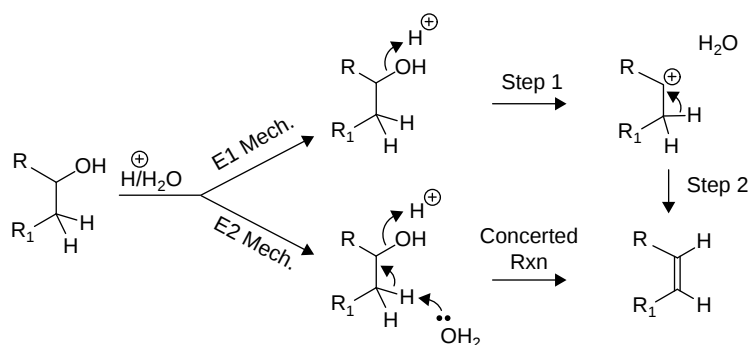


Figure 90 Acid-catalyzed alcohol dehydration, showing both E1 and E2 pathways.

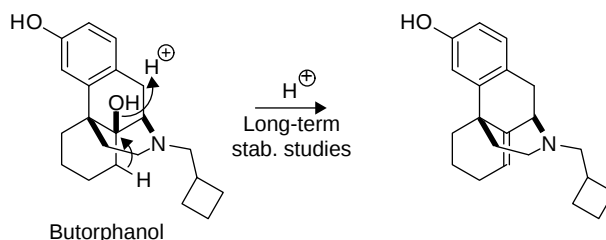


Figure 91 Acid-catalyzed alcohol dehydration of butorphanol.

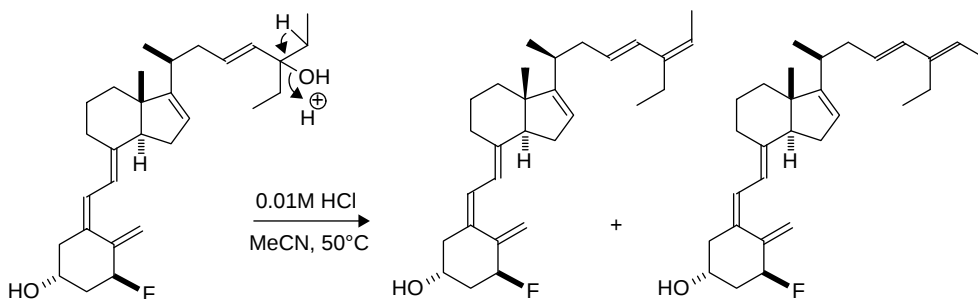


Figure 92 Vitamin D analog degradation under aqueous acidic conditions.

carbocation intermediate as the rate-determining step with loss of water whereas the E2 mechanism is second order with the base abstraction of a proton and loss of the leaving group occurring simultaneously (138,139). Acid-catalyzed elimination of a hydroxyl is seen in the case of butorphanol (Fig. 91).

An elimination reaction occurs under acidic conditions for the API vitamin D analog to yield the corresponding E/Z isomers as major degradation products (Fig. 92) (140).

Hydroxyls are not readily ionizable under normal pH conditions (e.g., pH 1–13). Hydroxyls have often been observed to participate in intramolecular cyclization reactions to form lactones from carboxylic acids, esters, and thioesters, especially if the lactone formed is a five or six-membered ring. This kind of degradation reaction is illustrated by degradation studies of the β -lactam antibiotic cephalothin (141) (Fig. 93).

Under (radical-initiated) oxidative stress conditions, an example of a hydroxyl group oxidizing to the corresponding ketone derivative has been described in the case of lovastatin

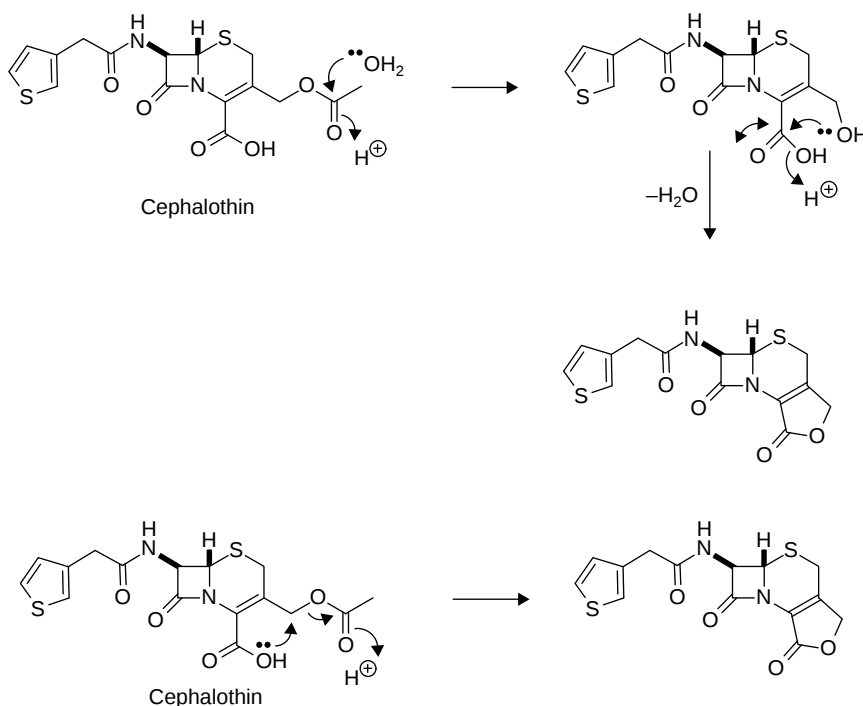


Figure 93 Lactone formation from intramolecular hydroxyl attack on a carboxylic acid.

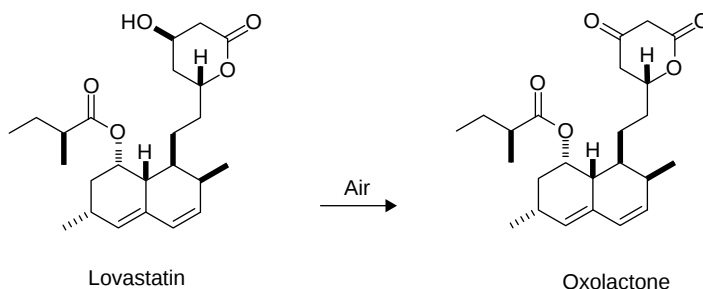


Figure 94 Lovastatin oxidation.

(Fig. 94) (142). This is somewhat unusual in that this hydroxyl would appear to be less oxidatively susceptible than other sites in the molecule (e.g., allylic tertiary sites with the potential for delocalization of the radical formed during autoxidation). Such an observation is an illustration that unpredicted reactions that can occur in degradation chemistry, especially in the solid state.

Tertiary hydroxyls can undergo several reactions under acidic conditions to form artifacts in degradation experiments. In acidic acetonitrile/water solutions, tertiary alcohols can undergo a Ritter reaction (143) to form amides (Fig. 95), resulting in a characteristic $M+41$ MW change. These compounds can form readily under dissolving solvent conditions and should be regarded as artifacts and not degradants.

It has also been observed that tertiary hydroxyls form chloro compounds (artifacts) under acidic conditions using HCl (Fig. 96); such reactions may or may not be artifacts, depending on whether they were formed during sample preparation or under the stress (or stability) conditions (e.g., from the chloride counterion of an HCl salt).

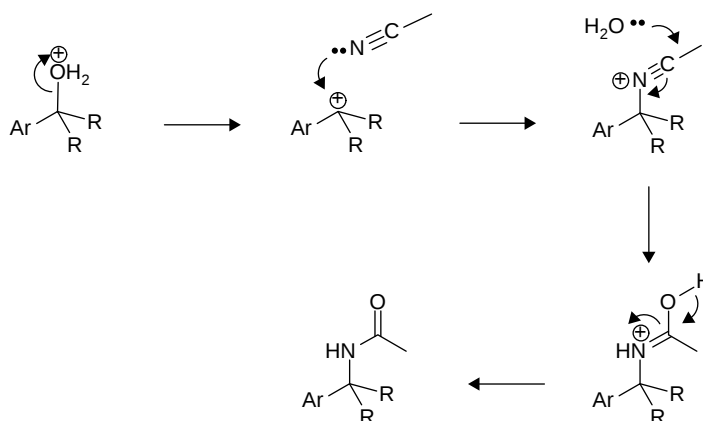


Figure 95 Ritter reaction of tertiary alcohols in acetonitrile to form amides.

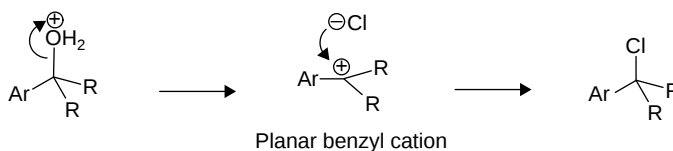


Figure 96 Degradation of tertiary hydroxyls to form chloro-derivatives under acidic (HCl) conditions.

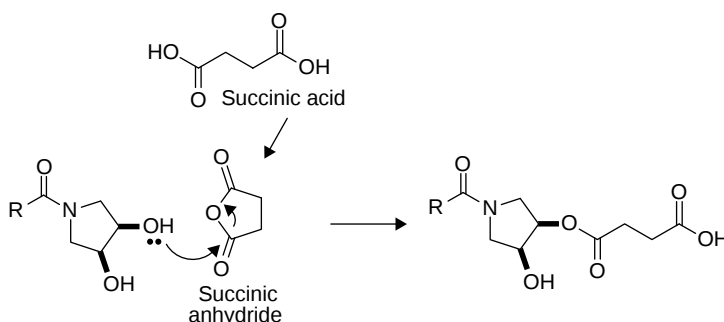


Figure 97 Esterification reaction of an API hydroxyl and succinic acid.

Ester formation with API hydroxyl groups has been observed for acid salts (e.g., succinic acid, citric acid, formic acid, acetic acid, etc) as well as excipients (e.g., stearic acid, magnesium stearate). See Figure 97 as an example of the reaction of a hydroxyl group with succinic acid (144).

Phenols

Phenols are known to undergo facile oxidation, and the oxidative chemistry has been studied extensively (145). The hydroxyl is strongly electron-donating into the phenyl ring, and is the key to the oxidizability of the ring. Abstraction of the hydrogen atom provides a particularly stable radical that can lead to reaction with molecular oxygen as shown in Figure 98. Deprotonation of the phenol at high pH to the phenolate anion greatly catalyzes the autoxidation process, allowing direct reaction with molecular oxygen (base-catalyzed autoxidation).

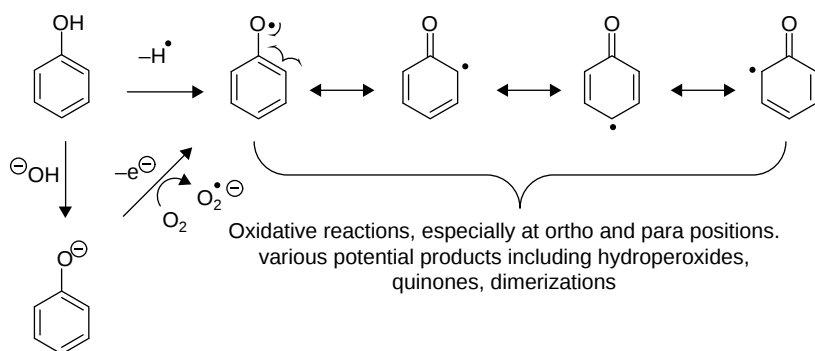


Figure 98 Simplified view of oxidative degradation chemistry of phenolic compounds.

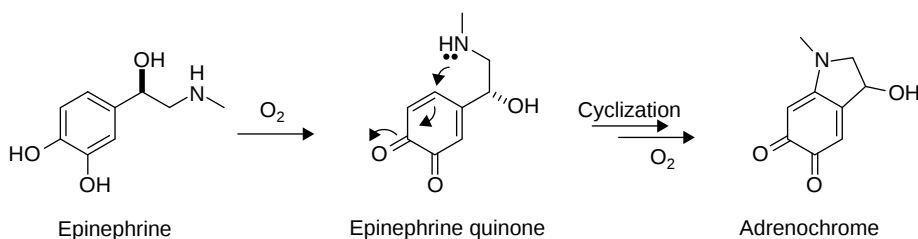


Figure 99 Oxidation of epinephrine to adrenochrome.

The phenolate anion is also an effective nucleophile and can react with electrophilic species at either the phenolic oxygen or the *ortho* or *para* positions.

The API epinephrine is an *o*-diphenol containing a hydroxyl group in the α -position that is easily oxidized by molecular oxygen (Fig. 99). Oxidation is proposed to occur through the transient formation of epinephrine quinone with subsequent formation of adrenochrome (146). This class of compounds (the adrenergics, including adrenaline and isoprenaline) also undergo this reaction to form adrenochrome upon photoirradiation in the aqueous solution (147).

A similar oxidation and intramolecular cyclization is observed for the *o*-diphenol levarte-renol (148).

Alkyl Halides

Alkyl and aryl halides are susceptible to hydrolysis leading to a hydroxyl plus the resulting halo acid (Fig. 100). For alkyl halides, the hydrolysis can occur via S_N1 (cationic intermediate, associated with a racemization if the center is asymmetric), S_N2 (direct nucleophilic attack with inversion of configuration) or by other mechanisms, but a detailed mechanistic study is beyond the scope of this chapter.

The susceptibility of alkyl halides to hydrolysis is a function of the halide (generally, $I > Br > Cl > F$). In contrast, the rates of aryl halide hydrolysis is generally $F > Cl > Br > I$. According to March, fluoro is generally a much better leaving group than other halogens $Cl > Br > I$ (but not

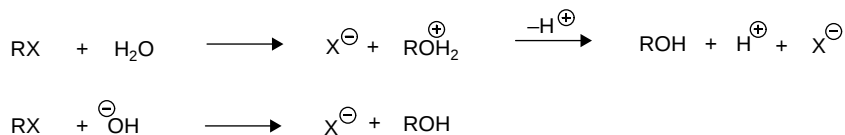


Figure 100 Alkyl halide hydrolysis (acid and base catalyzed).

always). Fluoro is the poorest leaving group when the second step of the S_NAr mechanism is rate determining (149). Aryl halides hydrolyze via an addition-elimination reaction.

The conformation of an alkyl halide can have a dramatic effect on its susceptibility for elimination. For example, if the halide is oriented antiperiplanar (90° torsion angle) or syn-periplanar (0° torsion angle) to a hydrogen on an adjacent carbon, the elimination of the haloacid is greatly favored. Hydrolysis of alkyl halides can be dramatically facilitated by the presence of a nitrogen or sulfur attached to the carbon alpha to the halide. This enhancement of solvolysis (e.g., 10–1000 times faster) is the result of intramolecular nucleophilic attack of the sulfur or nitrogen to form a cationic three-membered ring (an episulfonium ion in the case of sulfur or an aziridinium ion in the case of a nitrogen) as shown in Figure 101. Such neighboring

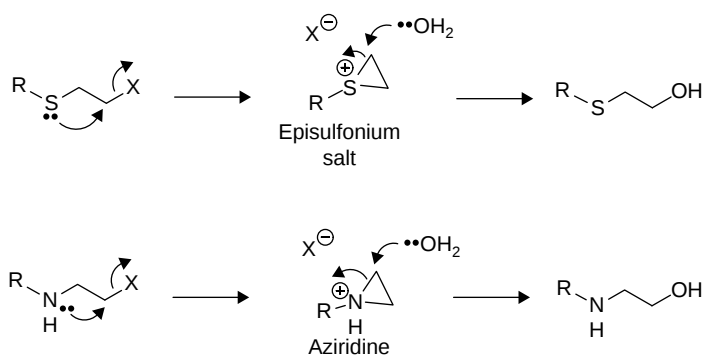
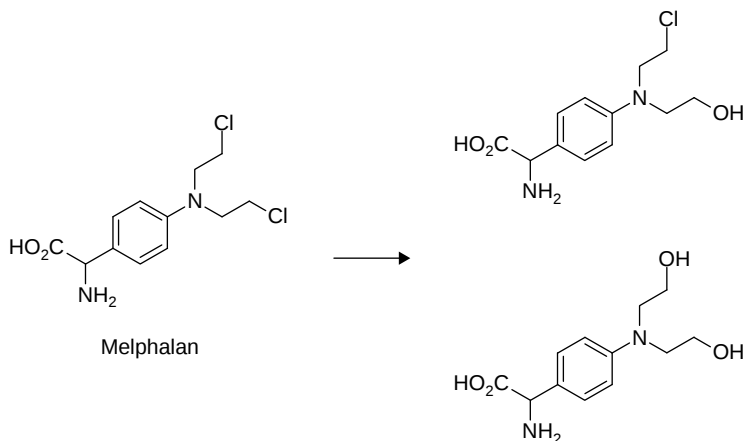


Figure 101 Neighboring sulfur or nitrogen group assistance of solvolysis of alkyl halides.



Proposed mechanism of hydrolysis

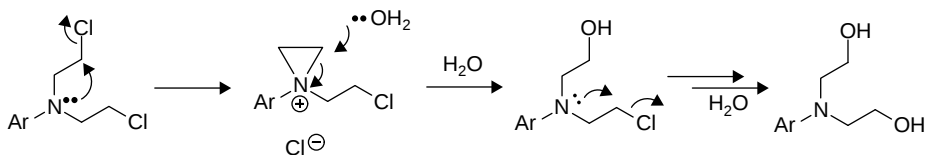


Figure 102 Hydrolysis of melphalan and proposed mechanism.

group assistance requires conformational flexibility in order to form the three-membered ring. The neighboring group assistance mechanism consists essentially of two S_N2 substitutions [one intramolecular (sulfur attack) and one intermolecular (water attack)].

Hydrolysis of the API melphalan is an example involving nitrogen group assistance of solvolysis of a dialkyl halide to form the dialkyl alcohol (Fig. 102) (150).

Hydrolysis of the dichloroacetamide functionality occurs in aqueous solutions with the API chloramphenicol under basic conditions. A primary route of decomposition for chloramphenicol

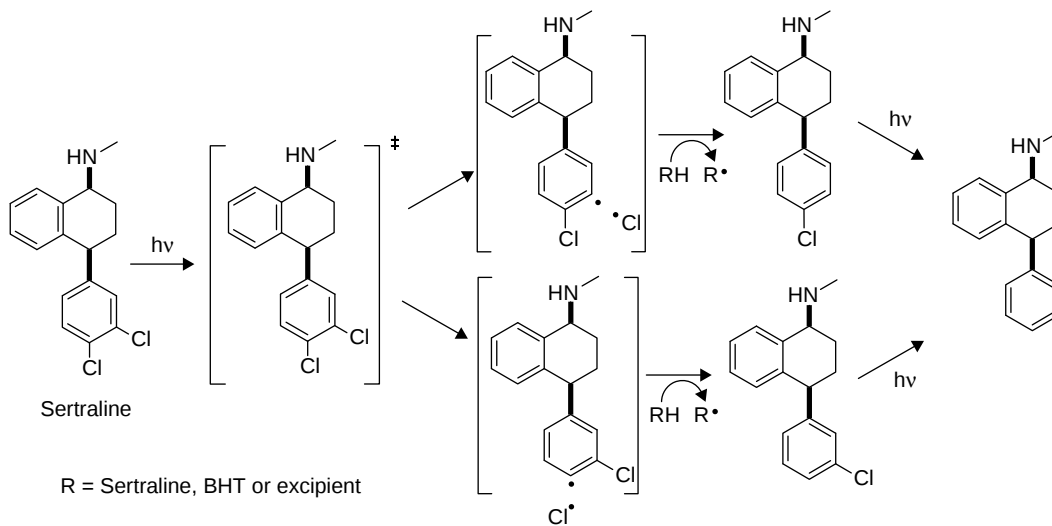


Figure 103 Sertraline photodegradation in solution.

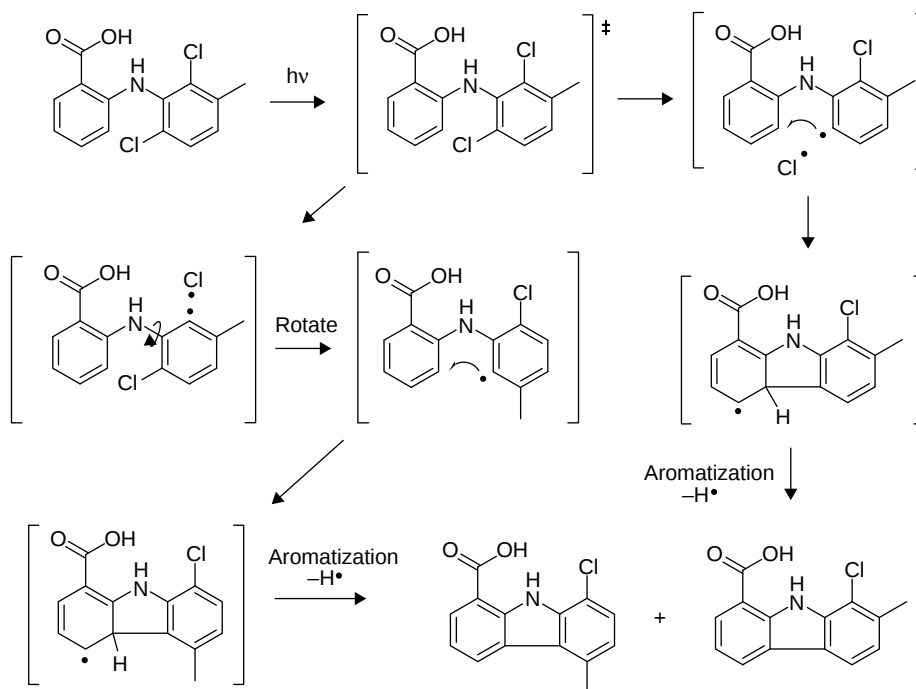


Figure 104 Meclofenamic acid photochemical induced dechlorination.

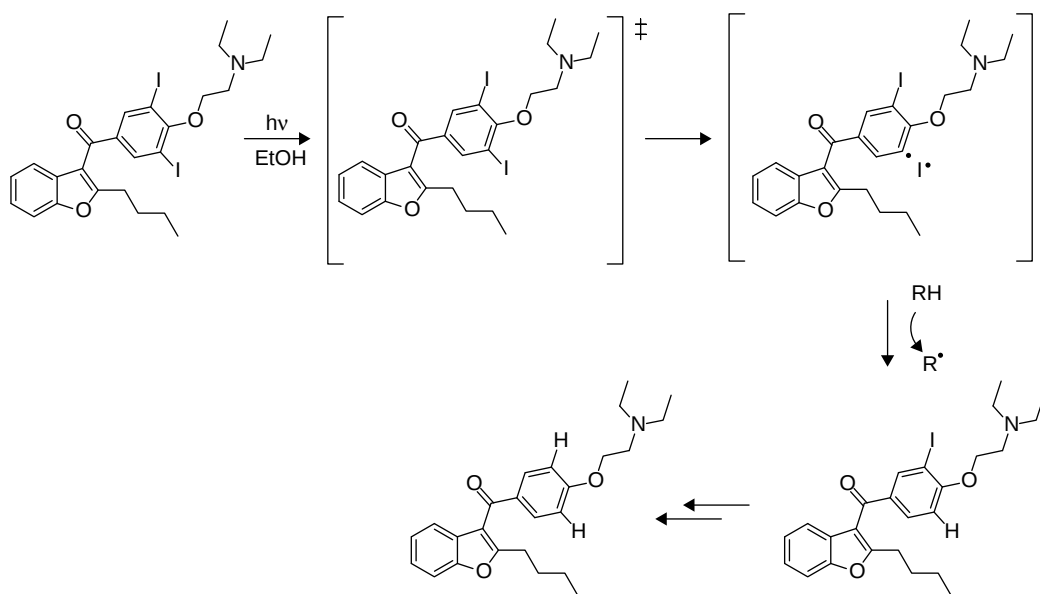


Figure 105 Amiodarone photochemical induced de-iodination.

in aqueous solution involves hydrolysis of the covalent chlorine of the dichloroacetamide functional group (151).

Aryl halides are often susceptible to photochemical degradation. As described in chapter 7 later in this book, cleavage of the C–X bond occurs with low quantum yield for aryl chlorides (152) higher quantum yields for aryl bromides and iodides (153) and high quantum yields for some aryl fluorides (e.g., fluoroquinolones) (154).

Aryl chlorides are photolabile to homolytic and/or heterolytic dechlorination. For sertraline hydrochloride, decomposition of the aryl dichloride moiety occurs in solution when exposed to light (ultraviolet and cool white fluorescent conditions, ICH Option 1). As shown in the following proposed mechanism, the major photochemical decomposition products include mono-chloro- and des-chloro-sertraline via homolytic cleavage (Fig. 103) (103).

Aromatic photodechlorination is also observed for the API meclofenamic acid (155). Meclofenamic acid undergoes photochemical dechlorination and ring-closure to carbazole products (Fig. 104).

The cardiac agent API amiodarone was observed to deiodinate sequentially upon photo irradiation in deaerated ethanol to yield the mono-iodo product and finally the des-iodo product (Fig. 105) (156). Formation of aryl radicals during the de-iodination process was supported by a spin trapping study.

Benzyl Groups

Benzyl groups are stable to most conditions but are susceptible to autoxidation as shown in Figure 106. The free radical process of autoxidation consists of a chain sequence involving three distinct types of reactions: initiation, propagation and termination (Fig. 107) (157). The initiation step produces a free radical to begin the chain reaction.

Using a radical chain initiator is a valid method of accelerating autoxidation. Radical chain initiating diazenes [e.g., azobisisobutyronitrile (AIBN)] undergo thermal bond homolysis to yield two radicals and molecular nitrogen. The resultant “initiator” radicals (R•) are highly reactive/high energy intermediates and react rapidly with an oxygen molecule to form peroxy radicals (ROO•). The addition of molecular oxygen to the “initiator” radicals (R•) to is

extremely fast and is probably diffusion controlled (the diffusion controlled rate in solution is approximately $10^9 \text{ M}^{-1} \text{ s}^{-1}$, depending on the solvent system). The resultant peroxy radicals ($\text{ROO}\bullet$) are relatively stable and longer-lived, compared to the initiator radical ($\text{R}\bullet$), and are able to react with a number of organic substrates. The resulting peroxy radical hydrogen atom abstraction from the API/excipient is typically the rate-determining step (k_p rate constant) in radical-initiated oxidation reactions. The predominate reaction of peroxy radicals, with respect to drug degradation, is the hydrogen atom abstraction from an API molecule or excipient to form API free radicals ($\text{API}\bullet$) and/or excipient free radicals ($\text{Excip}\bullet$). The API or excipient free radicals react rapidly with oxygen to form peroxy radicals as shown in the propagation step of Figure 107.

Termination of the autoxidation chain process occurs as peroxy radicals react with other radicals to yield nonradical products. The major termination reaction takes place through an unstable tetroxide intermediate. Primary and secondary tetroxides decompose rapidly by the Russell termination mechanism to yield three nonradical products via a six-membered cyclic transition state (Fig. 108). The decomposition yields the corresponding alcohol, carbonyl compound and molecular oxygen [formation of singlet oxygen is possible via this mechanism (158)], three nonradical products that terminate the chain process (159). Oxidations can be

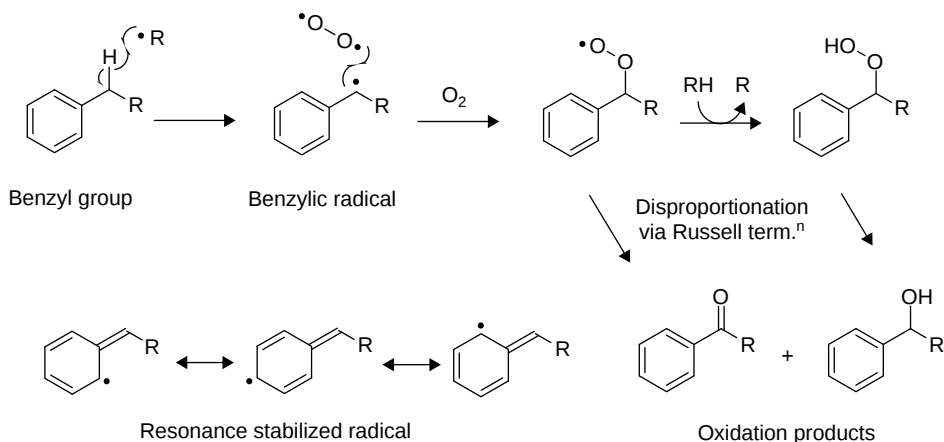


Figure 106 Oxidative degradation of benzyl groups.

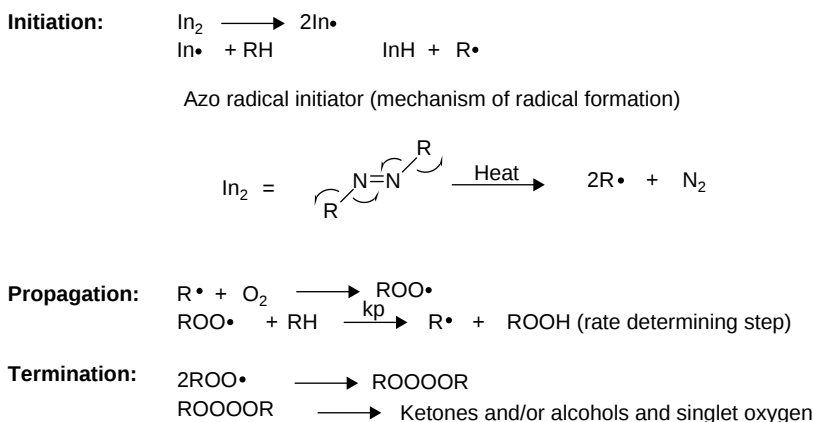


Figure 107 Autoxidation chain reaction.

catalyzed by peroxide-containing forming excipients (e.g., PEGs, Tween 80/Polysorbates, Povidone, etc.). These excipients contain polymeric chains of polyethylene units ($-\text{O}-\text{CH}_2-\text{CH}_2-\text{O}-$), and this ether based functional group is prone to radical catalyzed oxidation. These reactive radical intermediates can catalyze oxidative degradation.

This susceptibility of benzyl groups to autoxidation is due to the low bond dissociation enthalpy of the benzylic hydrogen; abstraction of the benzylic hydrogen results in a benzylic radical (a π delocalized radical), stabilized by resonance into the phenyl ring. Such resonance delocalization could also be provided by other aryl groups or by extended conjugation, (160) and therefore any sp^3 hybridized methine ($-\text{CH}-$), methylene ($-\text{CH}_2-$), and even methyl ($-\text{CH}_3$) group attached to an aryl group or to a group with extended conjugation, provides a favorable site for autoxidation to occur.

If the benzylic site is chiral and has labile hydrogen, epimerization reactions may also occur via radical catalyzed mechanism, especially if the benzyl group is involved in a photo-degradation pathway (e.g., the benzyl group is photoexcited via exposure to light). As shown in Figure 109, sertraline HCl API in solution degrades under photo conditions to the *trans*-sertraline product. This is proposed to form through a dibenzylic radical intermediate, formed presumably via the triplet-excited state in the dichlorophenyl ring. If the degradation chemistry is performed in an aqueous environment, hydroxyl addition can also occur. Additionally, chiral benzylic alcohols are likely to undergo racemization reactions under acidic conditions

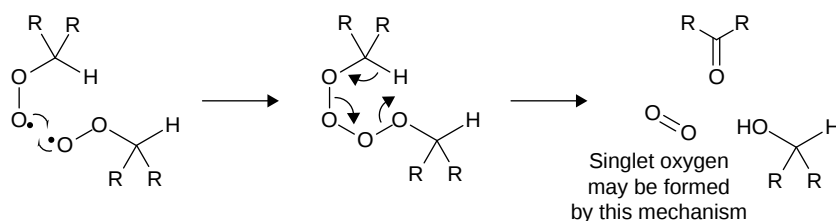


Figure 108 Russell termination mechanism.

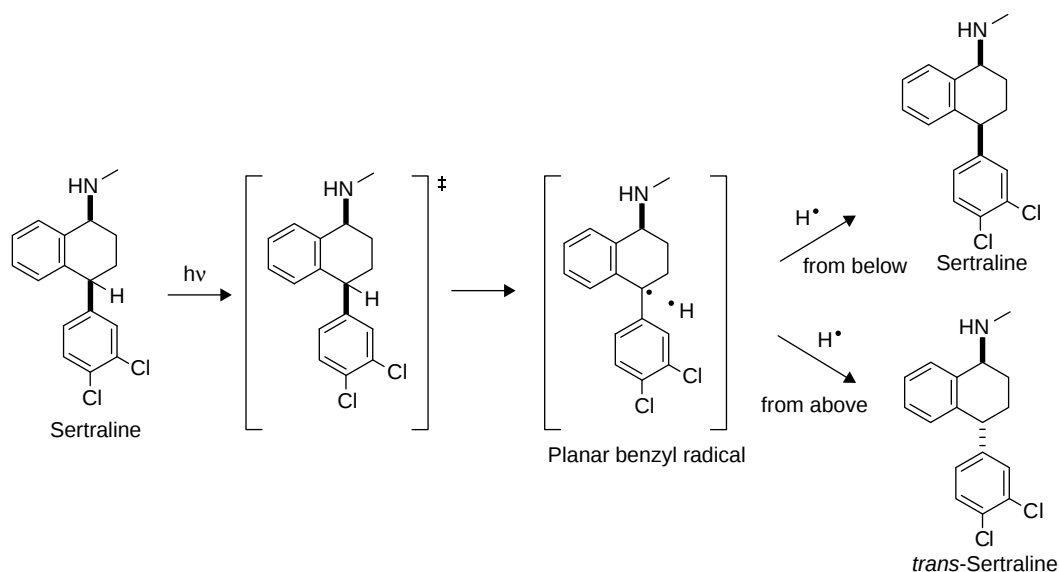
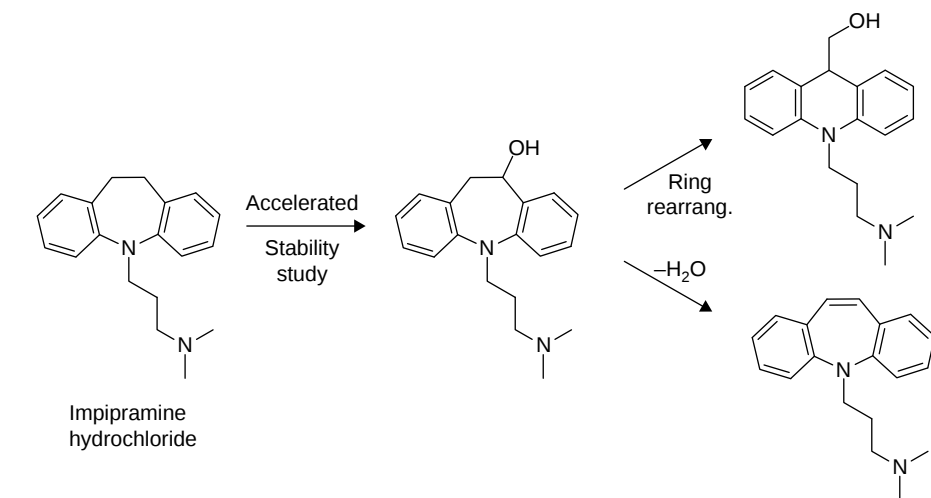


Figure 109 Sertraline HCl API epimerization at dibenzylic position to *trans*-sertraline.

via a cationic intermediate. Due to the low bond dissociation energy of the benzylic C–H bond and ease of radical formation, another reaction to keep in mind is potential dimerization of two molecules of the API at the benzylic center.

Compounds with a benzylic amine are particularly susceptible to hydrolysis and to radical initiated oxidation conditions. These compounds readily convert to the corresponding imine, which subsequently undergo hydrolysis to the primary amine and aldehyde derivatives.

Imipramine hydrochloride API undergoes oxidation at the benzylic site to form the corresponding benzyl hydroxyl compound (Fig. 110). Subsequent elimination of the hydroxyl occurs to give an extended conjugation in the molecule. By another pathway, the benzyl hydroxyl compound undergoes ring rearrangement from a seven- to a six-membered ring (161).



Proposed rearrangement mechanism

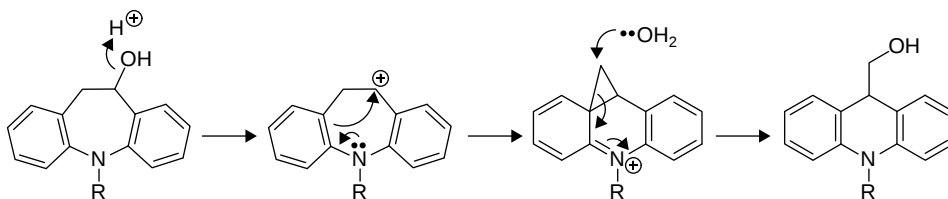
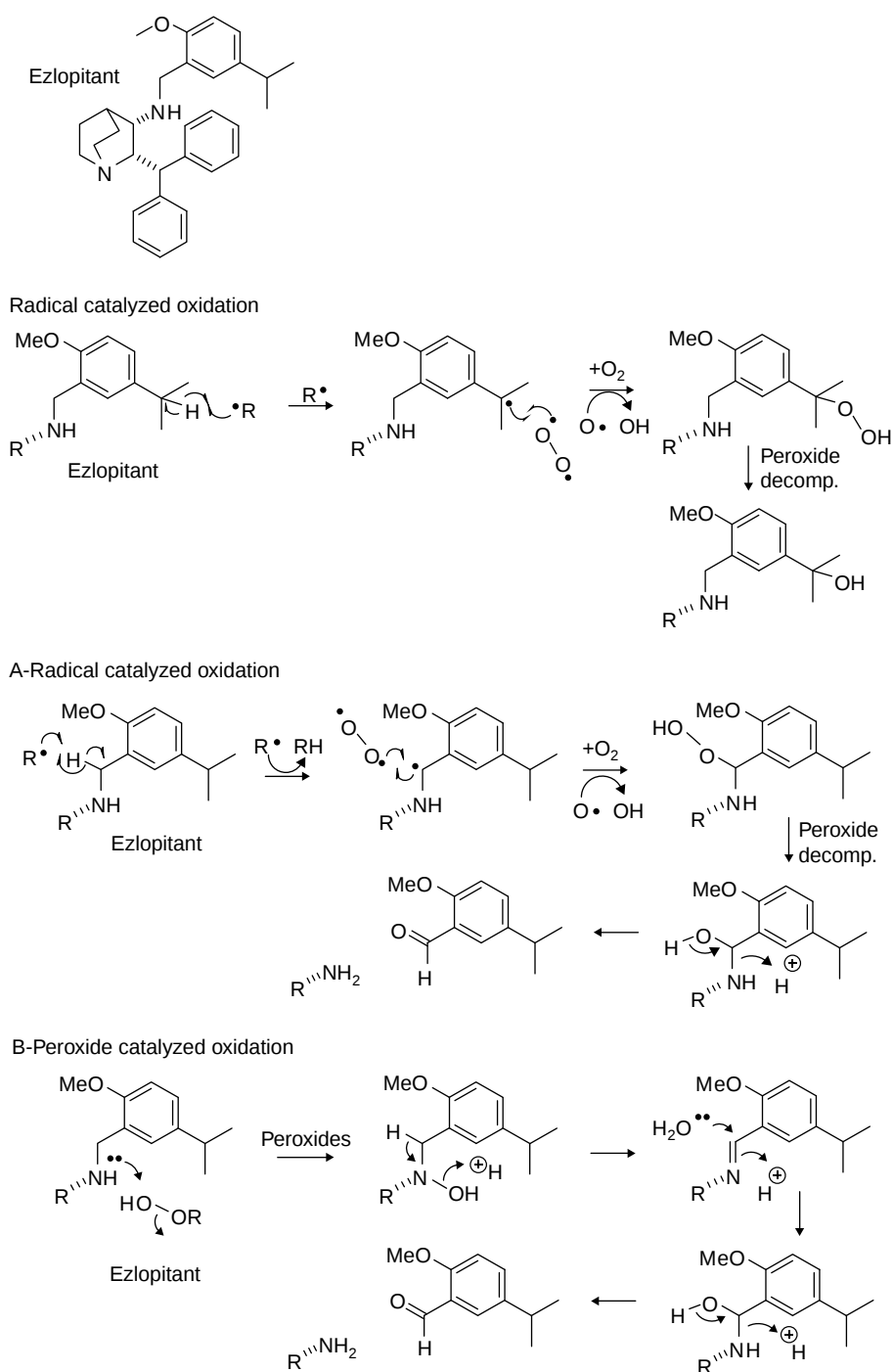


Figure 110 Imipramine hydrochloride benzylic oxidation chemistry. (Accelerated stability = 40°C/75% RH.)

For the following API candidate (Ezlopitant, Fig. 111), the isopropyl benzylic site is oxidized to the corresponding hydroperoxide (162). The hydroperoxide undergoes secondary degradation to the corresponding alcohol. The amine side chain can also degrade to form the free amine and the corresponding aldehyde; the reaction may be explained by two proposed oxidation pathways. According to proposed pathway A, a benzylic radical catalyzed oxidation would afford a benzylic hydroxyl group which happens to be formed adjacent to a nitrogen atom, affording a *hemi*-aminal. Collapse of this functional group would form the aldehyde and the amine. Pathway B would give the same degradation products; however, pathway B would produce an imine, which upon hydrolysis, leads to the amine and aldehyde. This potential for different degradation pathways to afford the same products illustrates the need to understand how the degradation products are formed in order to be able to design more stable formulations.

**Figure 111** Oxidation degradation pathways for ezlopitant.

The benzyl hydroxyl-containing API methoxamine hydrochloride was found to decompose in aqueous solution to the primary degradation product 2,5-dimethoxybenzaldehyde, presumably via a benzylic radical autoxidation pathway (Fig. 112) (163). The mechanism for this interesting degradation pathway was not proposed.

The benzyl hydroxyl containing API cyclandelate undergoes oxidation to the corresponding ketone 3,3,5-trimethylcyclohexyl phenylglyoxalate (Fig. 113), presumably via a benzylic radical autoxidation pathway (164). Low level benzylic oxidation is observed for the API ibuprofen. Oxidation to form the ketone derivatives at both benzylic sites to yield isobutylacetophenone and 2-(4-isobutrylphenyl)-propionic acid have been reported (165).

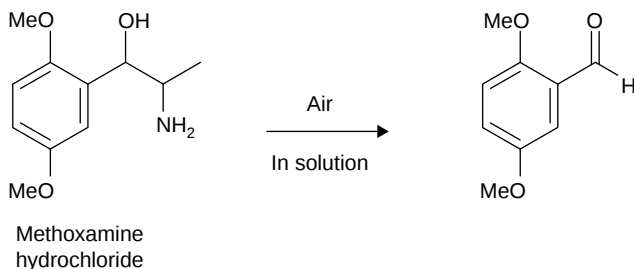


Figure 112 Methoxamine HCl oxidation.

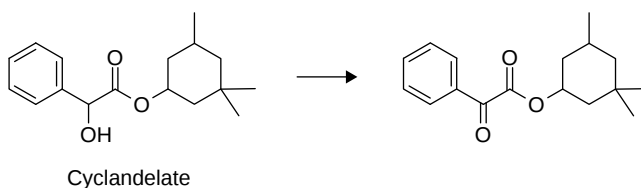


Figure 113 Cyclandelate oxidation.

Olefins

Olefins are compounds that contain one or more double bonds, and for the purposes of this book, are distinguished from “conjugated double bonds” as a separate section. Olefins can undergo a number of reactions that can be observed when subjected to stress testing conditions. When subjected to oxidation conditions, olefins (especially those that are conjugated with additional double bonds or that have heteroatoms in an allylic position) can undergo epoxidation and dihydroxy addition reactions (Fig. 114). Epoxidation followed by S_N2 reaction

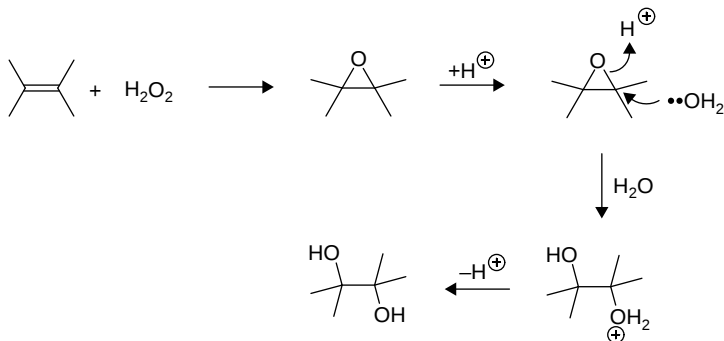


Figure 114 Epoxidation and dihydroxy addition of olefins.

resulting in antihydroxylation can occur by treatment with hydrogen peroxide and formic acid, common excipient impurities in drug product formulations (166).

In the case of the API dihydroergocristine methanesulfonate, autoxidation of the double bond of the heteroaromatic moiety yields the corresponding autoxidation products (Fig. 115) (167).

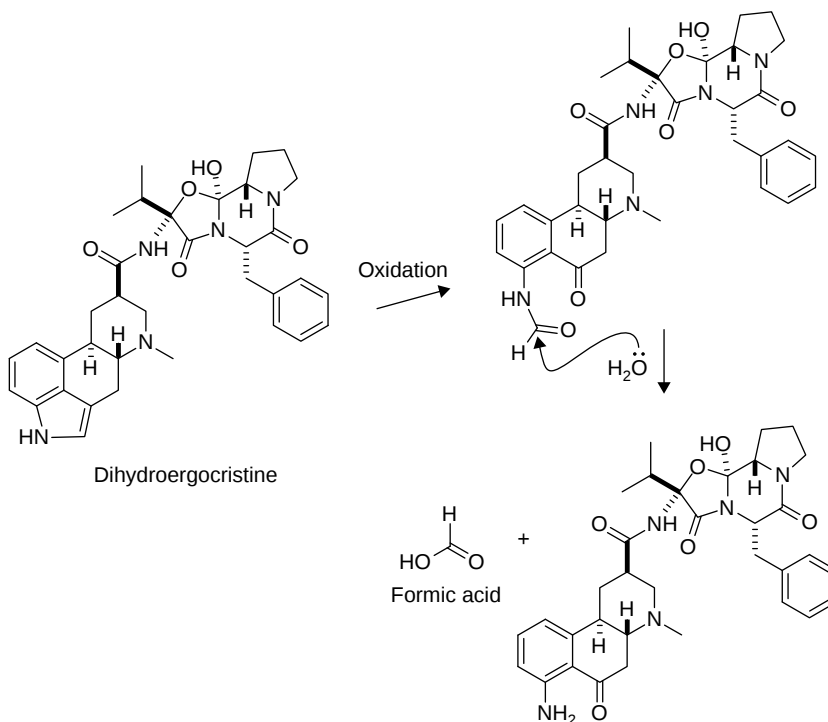


Figure 115 Autoxidation products of dihydroergocristine.

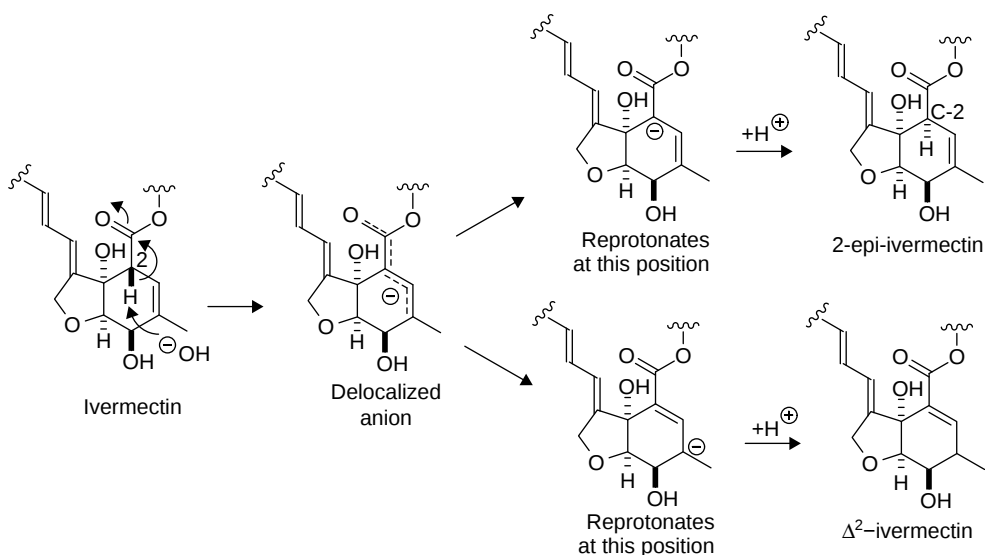


Figure 116 Alkaline hydrolysis products of API ivermectin.

Olefins are susceptible to isomerization and to migration. The API ivermectin undergoes isomerization under basic conditions due to the weak acidity of the hydrogen atom alpha to the ester group (i.e., C2). The allyl group on C2 (the carbonyl), affords the anion an additional degree of delocalization, increasing the acidity of this position even more. The two resulting degradants can be derived from the delocalized carbanion formed from dissociation of the proton at C2. Reprotonation at C2 generates the epimeric product and reprotonation at C4 generates the structural isomer (Fig. 116) (168).

The API thiothixene contains an olefin moiety that undergoes photo-oxidative olefin cleavage to the major decomposition product, the corresponding thioxanthone, through an endoperoxide intermediate (Fig. 117). The proposed mechanism of ketone formation occurs

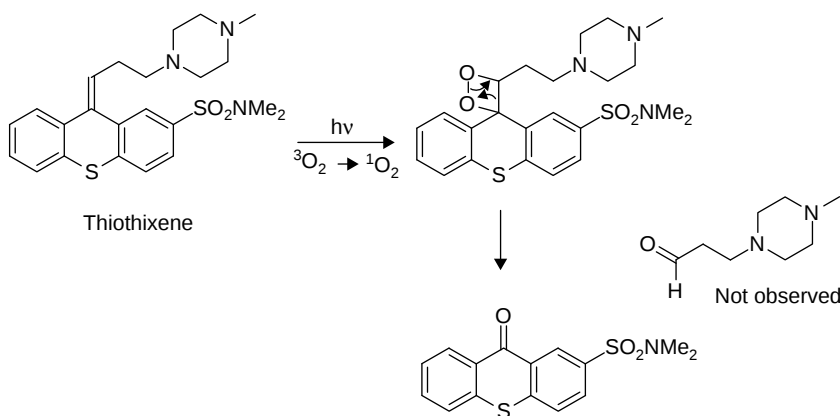


Figure 117 Photooxidation of thiothixene.

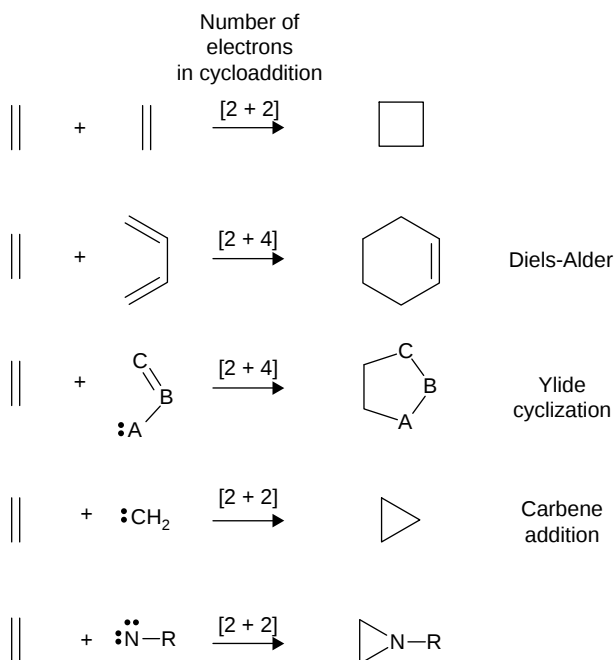


Figure 118 Cycloaddition reactions of olefins.

through (i) addition of singlet oxygen to the olefin, resulting in a dioxetane intermediate that then collapses to the thioxanthone degradant, or (ii) by the formation of a charge transfer complex with oxygen forming a hydroperoxide intermediate (169). Singlet oxygen is formed by energy transfer from the photoactivated API to ground state oxygen giving the higher energy singlet oxygen. Hence, the drug is the photoactivator for the formation of singlet oxygen.

For the diene containing API lovastatin, oxidation of the diene to the corresponding epoxide occurs in the presence of air (170).

Olefins are also susceptible to cycloaddition reactions (Fig. 118) (171). In particular, some olefin-containing APIs can dimerize with another molecule of API to form a [2+2] cycloaddition product under photo conditions (172). A classic example of such a [2+2] cycloaddition catalyzed by UV radiation is that of the nucleoside thymidine (173,174) (Fig. 119). These reactions are proposed to go through more than one mechanism: concerted, diradical, electron transfer, and radical ion pairs. The reaction can occur between neighboring thymidines on the same DNA strand (intrastrand) or between two different strands to form interstrand cross-links (for further discussion of nucleoside chemistry see chap. 15).

Olefins are also susceptible to photodegradation reactions other than cycloaddition reactions and react readily with singlet oxygen (175). The olefin bond is susceptible to *E(trans)*–*Z(cis)* isomerization (Fig. 120) as well as oxidation (Fig. 121) (176). Photochemical *E*–*Z* isomerization has a major role in photobiological systems and has practical applications in vitamin A and vitamin D industrial processes (177). Photoexcitation of the olefin produces a di-radical excited state, allowing rotation about the C–C bond, producing either the ground state *cis* or *trans* product. The sunscreen additive octylmethoxycinnamate undergoes *cis*–*trans* isomerization with high quantum yield as well as [2+2] cycloaddition reaction yielding a dimeric product (Fig. 122) (178). The *trans*-isomer was found to photoisomerise on irradiation at wavelengths greater than 300 nm. Photodimers were also separated and identified, and indicate that the sunscreen absorber can undergo [2+2] cycloaddition reactions with itself.

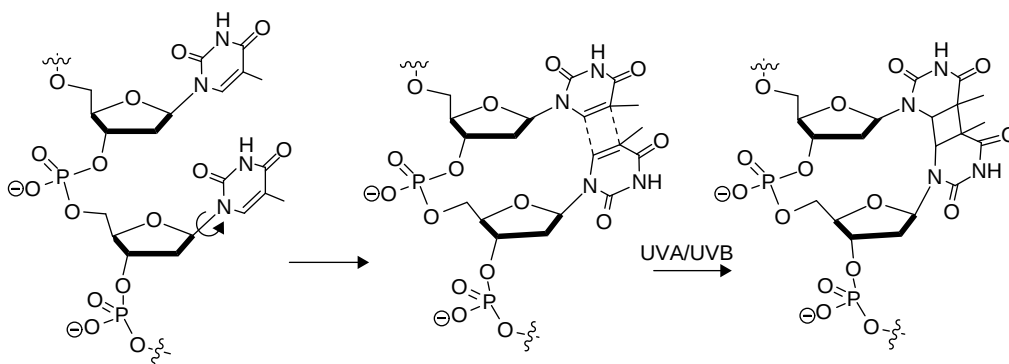


Figure 119 Illustration of thymidine [2+2] photocyclization reaction to form an intrastrand cyclobutane ring.

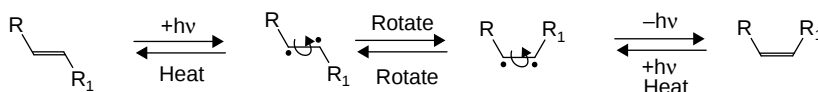


Figure 120 *Cis/trans* photoisomerization of double bonds.

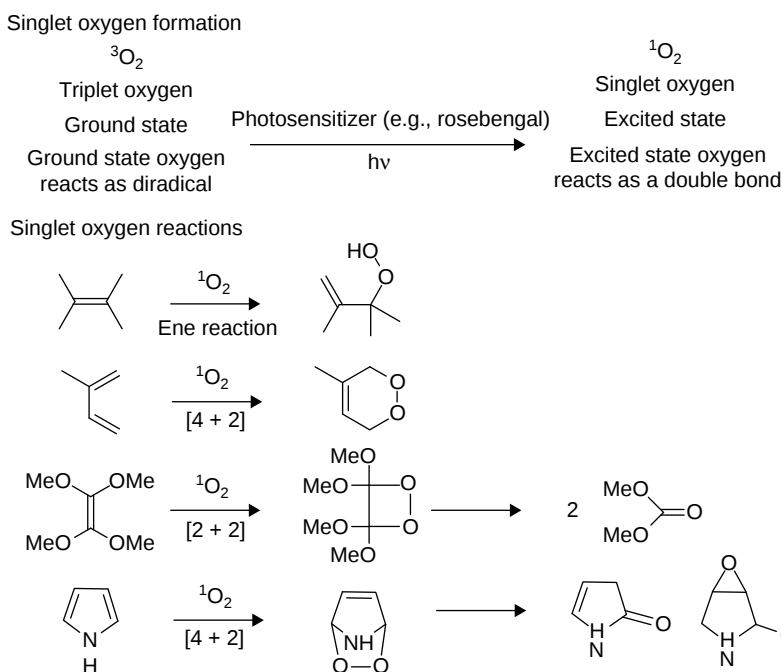


Figure 121 Olefin photochemistry reactions with singlet oxygen.

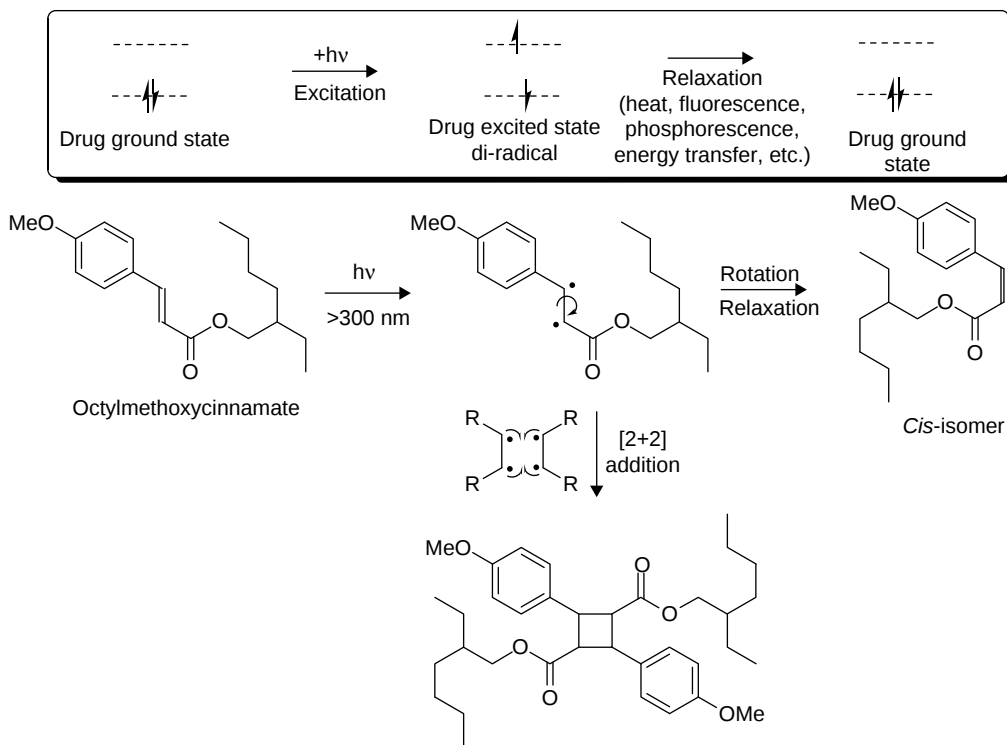


Figure 122 Trans-cis isomerization of octylmethoxycinnamate.

Allylic Groups

As mentioned previously for benzyl groups, an allylic center is also quite susceptible to autoxidation chemistry (Fig. 123). The allylic hydrogen has a weak C–H bond dissociation enthalpy (BDE) of approximately 89 kcal/mol, 10 kcal/mol lower than an aliphatic C–H BDE, due to the resonance stabilization energy of the resulting allylic radical (179).

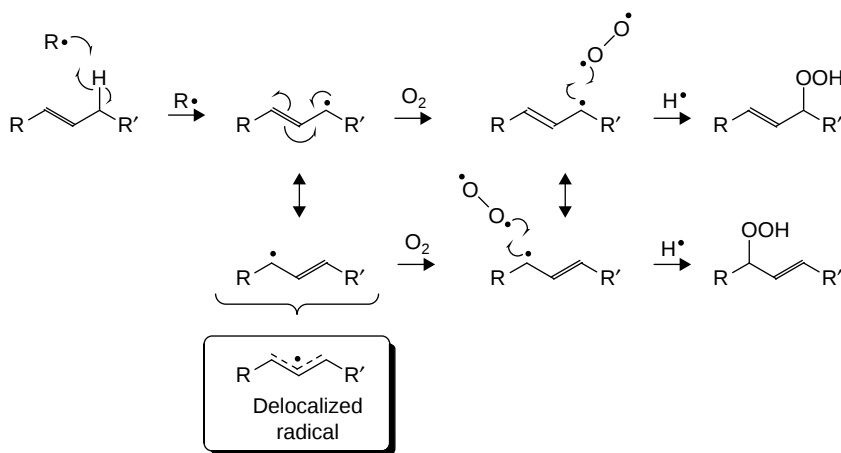


Figure 123 Allyl radical autoxidation mechanism.

Fatty Acids

FA s (which are also known as “lipids”) consist of a carboxyl function with an aliphatic chain. The aliphatic chain can be either saturated (i.e., no double bonds), monounsaturated (i.e., one double bond), or polyunsaturated (i.e., multiple double bonds). See Figure 124 for examples of FA structures.

Saturated FAs such as stearic acid are susceptible to degradation reactions typical of those for carboxyl groups. When protonated, e.g., pH < 4, the carboxyl group is electrophilic and can react with nucleophiles such as amines (to form amides) or alcohols (to form esters). When deprotonated, the carboxylate can act as a nucleophile. Saturated FAs are very stable to oxidative conditions. Unsaturated FAs, however, are very susceptible to autoxidation due to the presence of allylic or “doubly allylic” pentadienyl hydrogen atoms; in the case of polyunsaturated FAs, these doubly allylic hydrogen atoms can be abstracted to form a radical that is stabilized by delocalization over five carbon atoms as discussed immediately above (Figs. 107 and 108 for a detailed mechanism of the autoxidation chain process). Polyunsaturated FAs readily give rise to radicals under ordinary atmospheric storage conditions, trapping molecular oxygen and giving rise to hydroperoxides, hydroxyls, aldehydes, endoperoxides, epoxides, and even cyclized prostaglandin-like compounds (as shown in Fig. 125 for arachidonic acid) (180). Autoxidation of arachidonic acid yields six different peroxy products, and four of these peroxy radicals can undergo intramolecular cyclization to the dioxolane shown in the figure. The cyclization products can further degrade to multiple products via three main pathways: (i) substitution homolytic intramolecular (SH_i), (ii) cyclization (k_c), and (iii) further oxidation (O₂). Such autoxidation processes have been documented both in vitro and in vivo in the case of arachidonic acid to form so-called isoprostanes (181,182,183). The formation of numerous metabolic products from arachidonic acid has been termed the “arachidonic acid cascade” (184).

Vitamin E is effective as an antioxidant in arachidonate autoxidation, trapping the kinetic peroxy radical product before cyclization can occur. Adding Vitamin E in arachidonate autoxidation results in reducing radical cyclization products and forming the kinetic product distribution, six simple diene *trans*/*cis*-hydroperoxides.

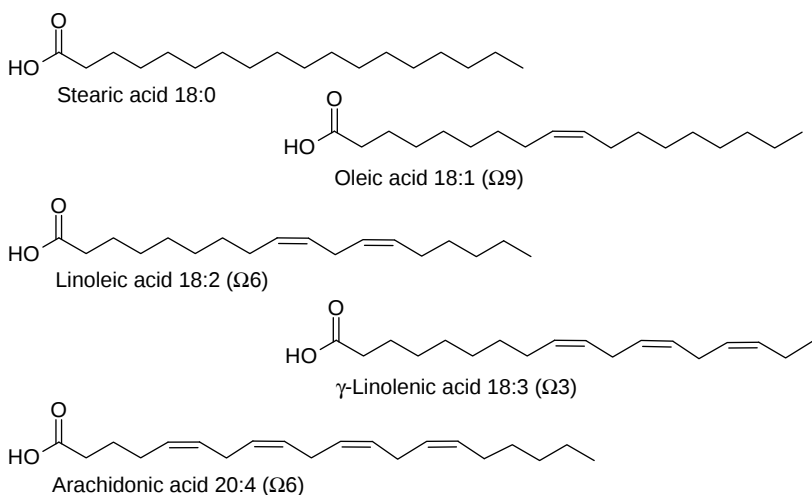


Figure 124 Structures of some common fatty acids.

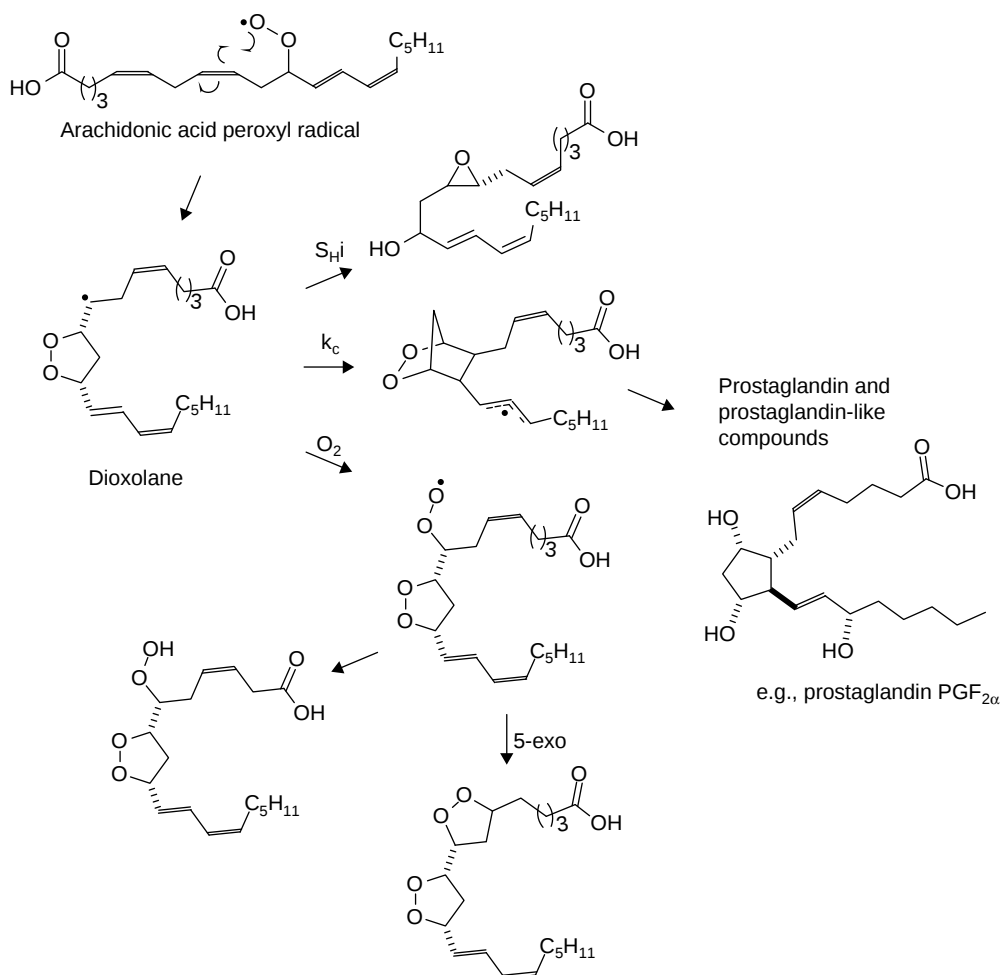


Figure 125 Overview of the complex autoxidation pathways of arachidonic acid.

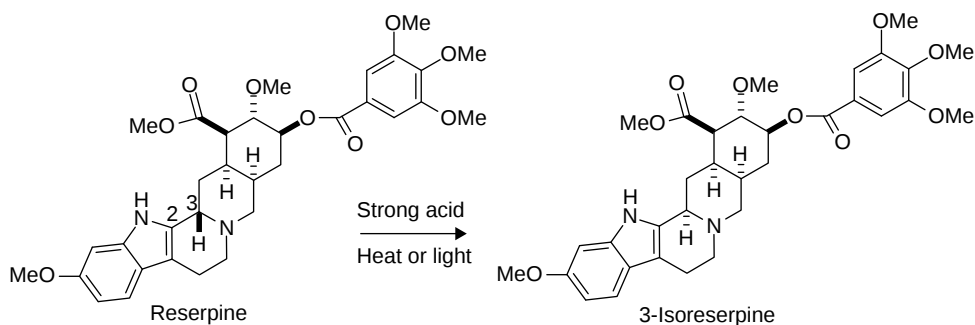
Racemization/Isomerization, Ring Transformations, and Dimerization**Racemization of API Chiral Centers**

Racemization reactions of chiral centers involve a planar intermediate reaction center (e.g., carbon-centered radical, cation, or anion) where the reacting molecule can approach the planar reaction center either from one side of the planar surface or the other side resulting in either partial or complete racemization of the chiral center.

Epimerization of the API reserpine to 3-isoreserpine occurs readily in strong acid solution but has also been observed in solution using heat or photolytic conditions (Fig. 126). The epimerization in this example has been shown to be initiated by protonation of C-2 following ring opening to yield an intermediate in which C-3 is planar and oriented for efficient ring closure to 3-isoreserpine (185). As has already been discussed (Fig. 116), the chiral center at C-2 of ivermectin undergoes epimerization under basic conditions.

Racemization of brinzolamide to the S isomer occurs under heat and light (pH independent) conditions (Fig. 127). This can occur via a radical mechanism to form a resonance stabilized planar radical with hydrogen atom addition occurring on both sides of the planar carbon centered-radical to racemize the stereocenter, analogous to the sertraline example discussed previously (Fig. 103) (186).

Epimerization also occurs under basic conditions for the lactone-containing API pilocarpine, which has a chiral center α to the carbonyl (Fig. 128) (187).



Proposed mechanism of epimerization

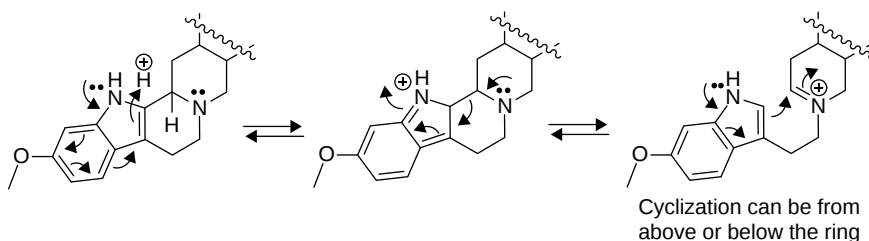


Figure 126 Racemization of reserpine to 3-isoreserpine.

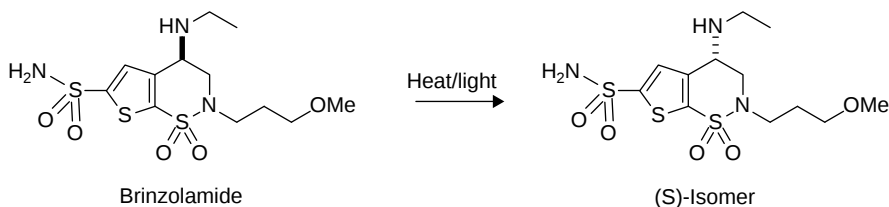


Figure 127 Racemization of brinzolamide.

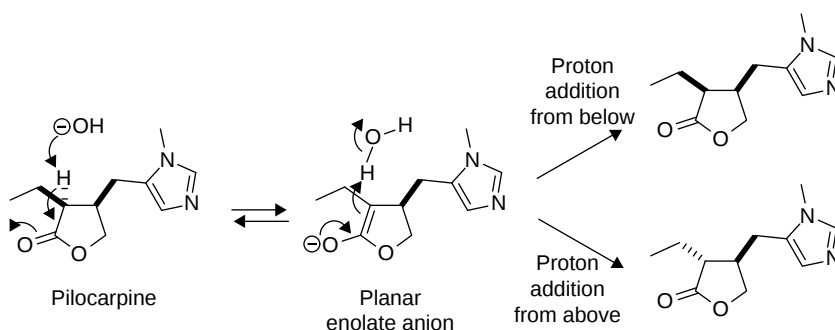


Figure 128 Hydrolysis and racemization mechanism of pilocarpine.

Ring Transformations

Ring transformations are common in pharmaceuticals. The API lorazepam is a benzodiazepine, which has a seven-membered nonaromatic ring with two nitrogens, and can lose a molecule of water and rearrange with the driving force being formation of a six-membered aromatic pyrimidine ring (Fig. 129) (188).

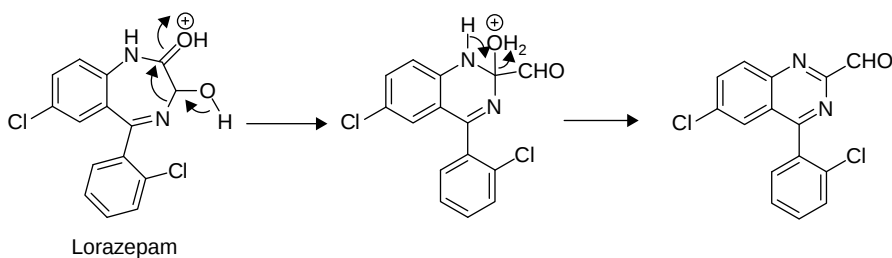


Figure 129 Lorazepam ring transformation.

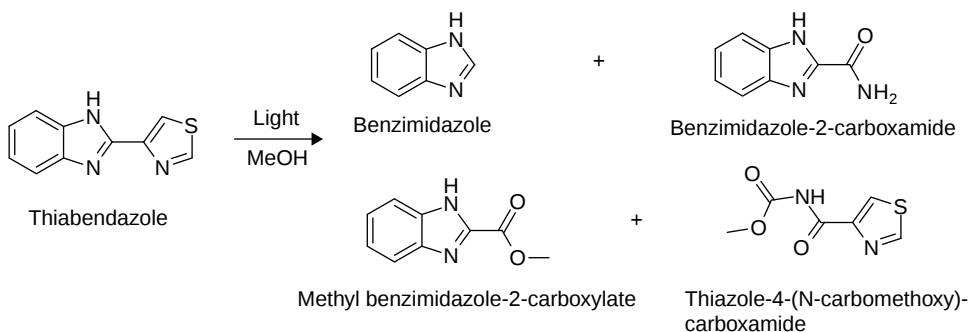


Figure 130 Thiabendazole photodegradation.

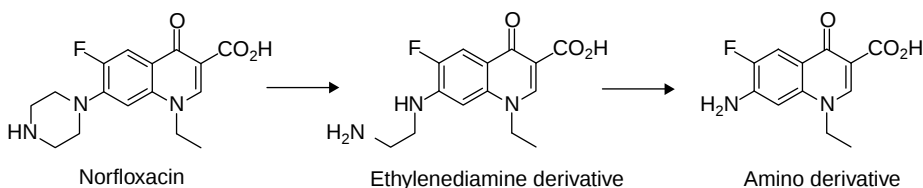


Figure 131 Norfloxacin piperazine undergoes photo-induced ring cleavage to ethylenediamine.

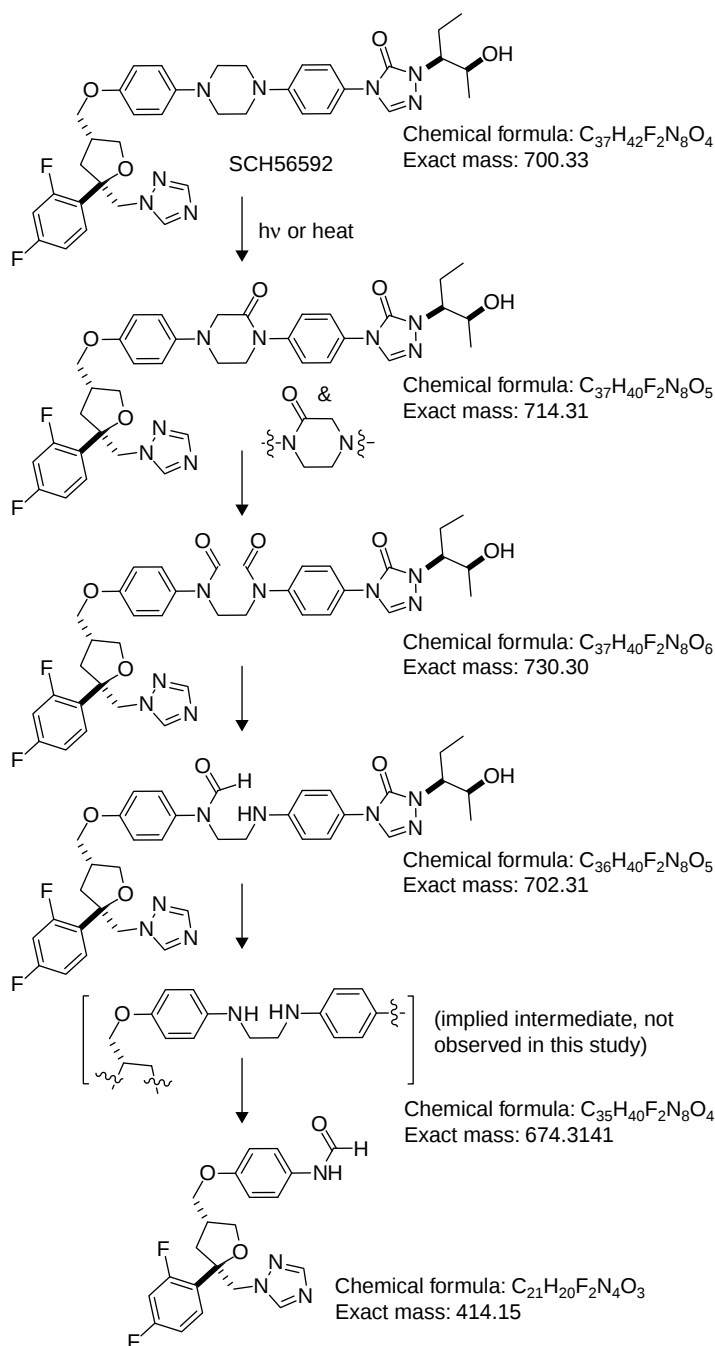


Figure 132 Photo and heat-induced degradation of the piperazine ring of SCH56592.

Imidazole and thiazole rings have demonstrated instability under photolytic conditions. For example, the API thiabendazole undergoes cleavage of the thiazole ring to form benzimidazole-2-carboxamide and benzimidazole as well as cleavage of the imidazole ring to form thiazole-4-(*N*-carbomethoxy)-carboxamide (Fig. 130) (189).

The API norfloxacin contains a piperazine ring that undergoes degradation under photolytic conditions in the solution and solid state to form the ring-opened ethylenediamine derivative and amino derivative (Fig. 131). Additional degradants observed in the solid state include the amino and formyl derivatives (190). This de-alkylation of piperazine rings is a common occurrence in drug degradation. For example, this degradation pathway was documented in the case of SCH 56592 under the stress conditions of either elevated temperature or photolysis (191). The structures determined during the study of SCH 56592 are shown in Figure 132, illustrating the presumed degradation pathway.

The APIs dipivefrin and epinephrine undergo ring formation when subjected to basic conditions to form adrenochrome (Fig. 99) (192).

Isomerization Reactions

The API etoposide contains a strained *trans*- γ -lactone ring that undergoes acid and base catalyzed degradation. Under basic conditions, the degradation of etoposide occurs through epimerization of the *trans*- γ -lactone ring to the *cis*- γ -lactone ring, occurring through a planar enol intermediate. Secondary degradation of the *cis*-etoposide then occurs to the *cis*-hydroxy acid (Fig. 133) (193).

Cephalosporin antibiotics will undergo isomerization of the double bond from the Δ^3 -position to the Δ^2 -position (Fig. 134), especially when the 4-carboxyl group is esterified (e.g., to

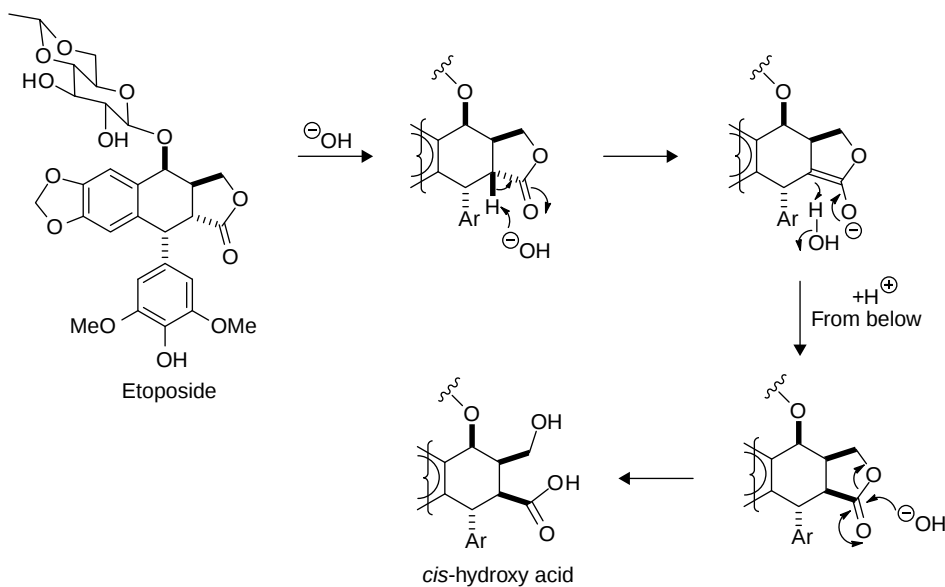


Figure 133 Etoposide epimerization.

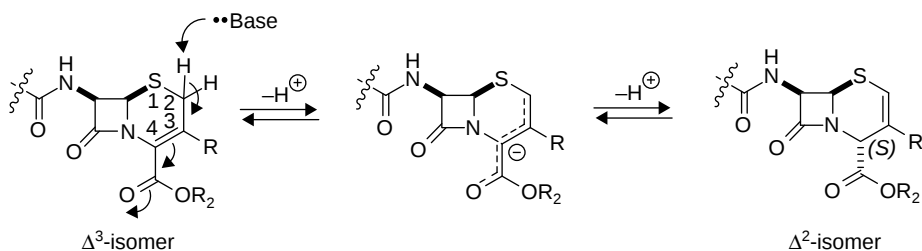


Figure 134 Cephalosporin isomerization of the olefin Δ^3 -position to the Δ^2 -position.

enhance bioavailability) (194). The isomerization reaction is subject to general and specific base catalysis in both directions (195). The reaction also occurs (although at a much slower rate) during either solution or solid-state degradation of nonesterified cephalosporins as in the case of cefaclor (28a,b). In the case of cefaclor, the protonation at C4 occurred stereospecifically to give the 4*S* configuration (proton is β).

Dimerization

Many compounds will undergo dimerization reactions: those containing olefins, alcohols, phenols, and carboxylic acids (or other carbonyl chemistry). Indoles have been shown to dimerize under acidic conditions. The dimerization is presumed to occur as shown in Figure 135 via protonation at C3 and nucleophilic attack of a second indole on C2.

Phenols have been shown to dimerize, usually at the *ortho*-position, under free radical-initiated oxidative conditions; the case of propofol, dimerizing at the *para* position due to steric reasons, is shown in Figure 136 (196).

Nalidixic acid API undergoes dimerization under elevated temperature to decarboxylate and produce a dimeric structure (Fig. 137) (197).

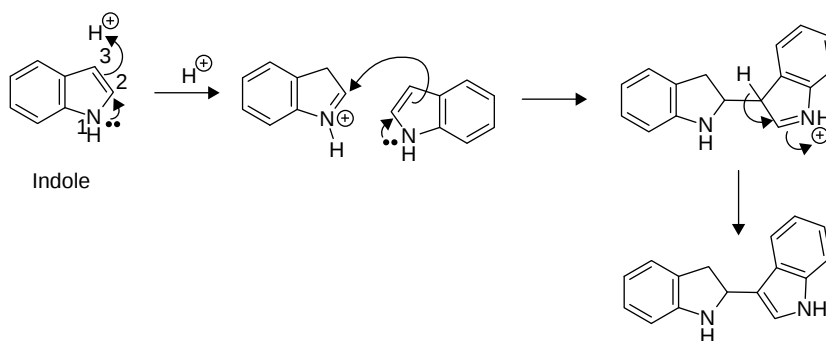


Figure 135 Dimerization mechanism for indoles under acidic conditions.

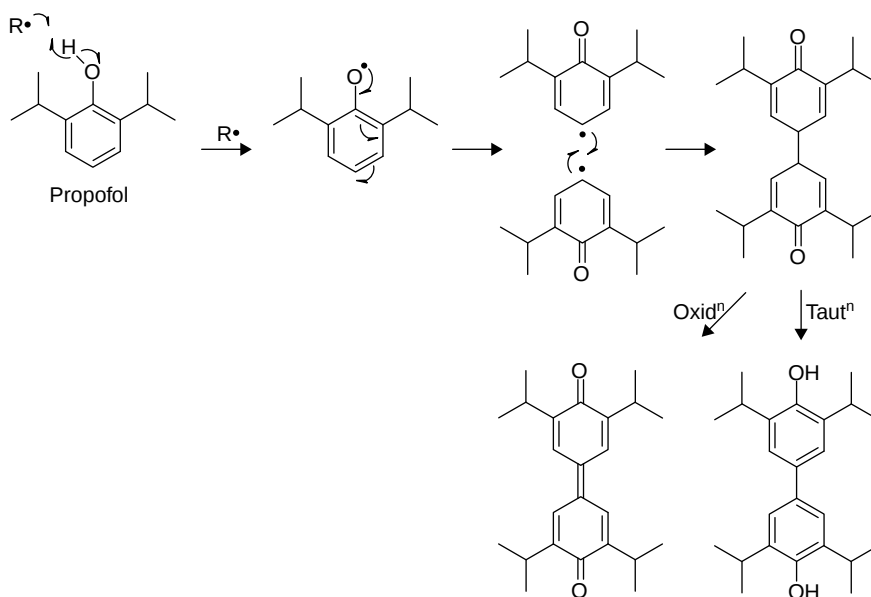


Figure 136 Dimerization of propofol.

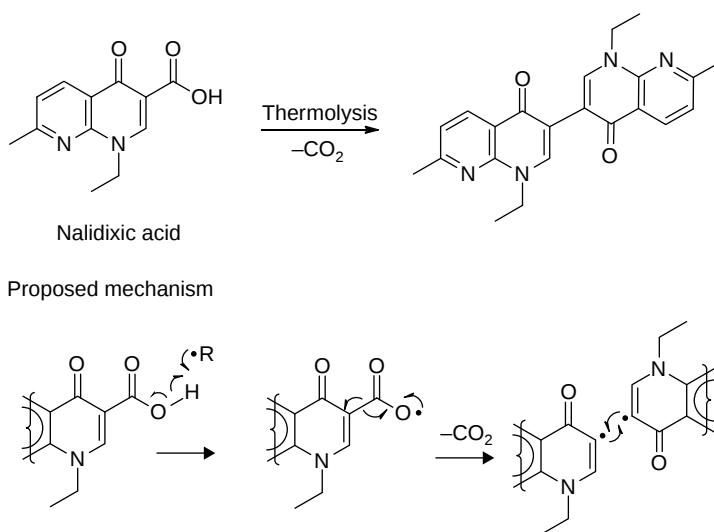


Figure 137 Nalidixic acid decarboxylation and dimerization.

Carbohydrates, Sugars

A comprehensive or thorough discussion of carbohydrate chemistry is beyond the scope of this chapter, but some discussion is warranted. A simple sugar is a straight-chain aldehyde or ketone that has alcohol functional groups on each of the remaining carbons. The aldehyde or ketone functional groups of a simple sugar can interact intramolecularly with an alcohol, forming a cyclic hemiacetal, either five- or six-membered rings containing one oxygen, furanose or pyranose forms, respectively.

In aqueous solution, monosaccharides (containing five or more carbon atoms) occur in ring (or cyclic) forms (198). These molecules are known as pyranoses (six-member ring) or furanoses (five-member ring) since they resemble pyran and furan, respectively (as shown in

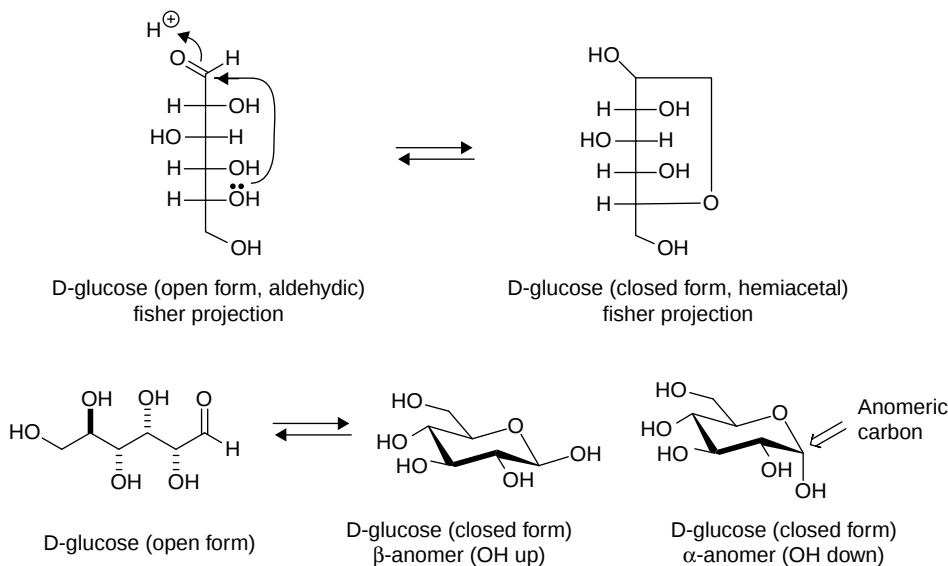
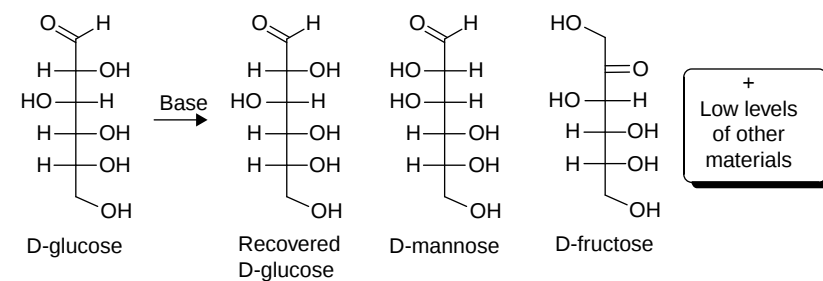


Figure 138 Glucose open chain (aldehydic form) and cyclic pyranose form.



Proposed glucose degradation mechanisms

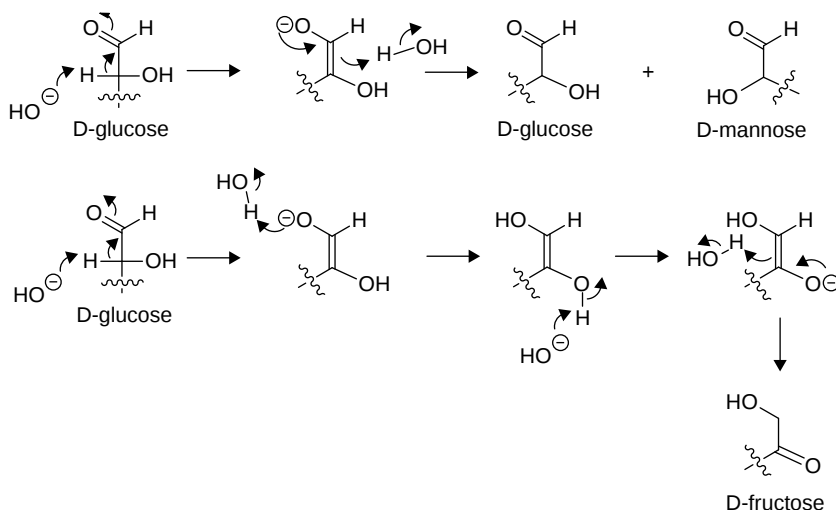
**Figure 139** D-Glucose reaction under basic conditions.

Fig. 138 for glucose). The rings form as a result of the aldehyde (or ketone) reacting with a hydroxyl group further along the chain (usually at the penultimate carbon) and forming a hemiacetal (or hemiketal) link. This is a general hemiacetal (hemiketal) reaction where aldehydes (ketones) combine with alcohols.

An extra chiral center is produced at the hemiacetal carbon (former aldehydic carbonyl carbon). The hydroxyl group can be either below (α) or above (β) the plane of the ring structure. Monosaccharides that differ only in the configuration of the groups at the hemiacetal carbon are known as *anomers*. The hemiacetal carbon is known as the anomeric carbon (Fig. 138).

Sugars with a free hydroxyl on the anomeric carbon (hemiacetals) are known as reducing sugars because they can open to the aldehydic form and then be readily oxidized to the carboxylic acid. The free anomeric carbon is often called the reducing end because when it is oxidized to a carboxylic acid it effectively reduces the other compound or atom. Nonreducing sugars are simple sugars that have an ether instead of a hydroxyl bond present at the anomeric carbon so that the sugar cannot be readily oxidized. Reducing sugars include lactose, fructose, glucose, and maltose. Nonreducing sugars include cellulose, sucrose, trehalose, and mannitol (mannitol is an alditol having all hydroxyl groups and therefore no cyclic/aldehydic forms).

In base, aldoses and ketoses rapidly equilibrate to mixtures of sugars (Fig. 139) (199). Most sugars react with alcohols under acidic conditions to yield cyclic acetals (glycosides). Glycoside formation, like acetal formation, is catalyzed by acid and involves cationic intermediates (Fig. 140).

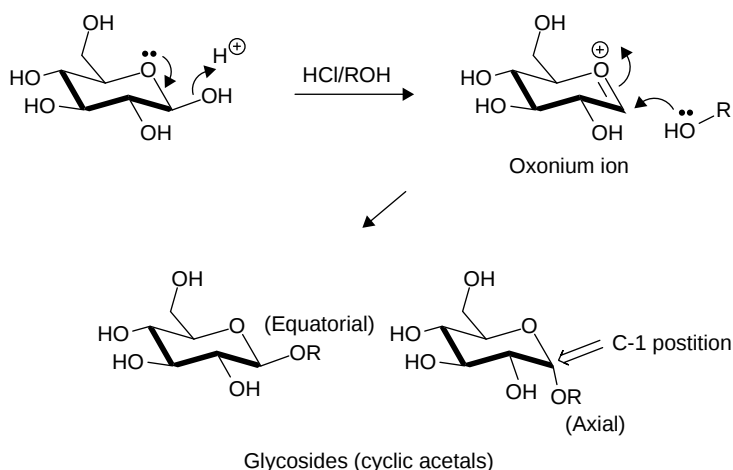


Figure 140 Reaction of sugars with alcohols to yield glycosides under acidic conditions.

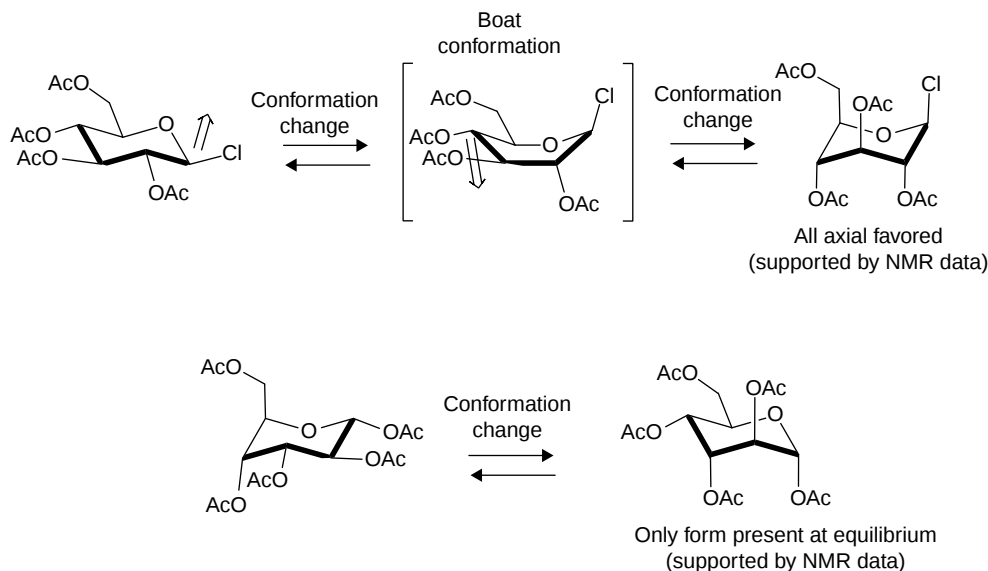


Figure 141 Equilibria in compounds that exhibit the anomeric effect, favoring the axial position for polar groups attached to the anomeric carbon.

In a six-membered ring, an alkyl group located on a carbon α to a heteroatom prefers the equatorial position (as expected), but a polar group (such as hydroxyl or $-OR$) prefers the axial position (known as the anomeric effect), and this is the reason for the greater stability of α -glycosides over β -glycosides (200). For example, pyranose sugars substituted with a polar electron-withdrawing group such as halogen or alkoxy at C1 are often more stable when the substituent has an axial orientation rather than an equatorial position (201).

The magnitude of the anomeric effect (202) depends on the substituent with the effect decreasing with increasing dielectric constant of the environment. In Figure 141, the tri-O-acetyl- β -D-xylopyranosyl chloride anomeric effect of the single chlorine drives the equilibrium to favor the conformation with the three-acetoxy groups in the axial positions. From a molecular orbital viewpoint, the anomeric effect results from an interaction between the lone pair

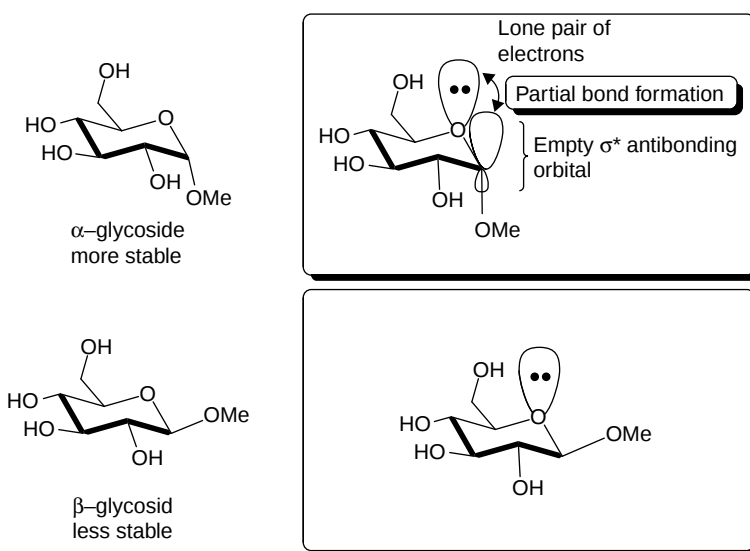


Figure 142 Anomeric effect, stability of α -glycosides over β -glycosides.

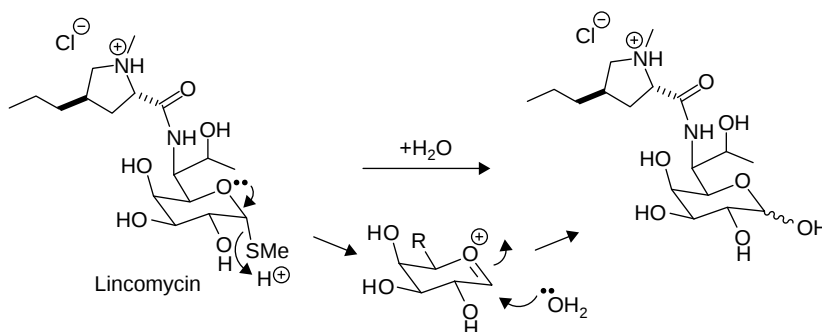


Figure 143 Lincomycin HCl thioglycoside hydrolysis at the carbohydrate anomeric center.

electrons on the pyran oxygen and the σ^* (empty antibonding) orbital associated with the bond to the C1 substituent, providing a stabilizing effect (Fig. 142).

Anomeric center reactivity has been observed for the API lincomycin hydrochloride. Lincomycin HCl contains a carbohydrate portion that undergoes thioglycoside hydrolysis at the carbohydrate anomeric center (Fig. 143) (203).

Nucleic Acids

Nucleic acids, similar to peptides and proteins, can possess not only primary structure but also secondary and tertiary structure. Oligomeric and polymeric nucleic acid structures can form the classic double helix duplex structure via hydrogen-bonded base pairing. The discussion here will focus only on a few of the major degradation pathways of the primary structure. More extensive reviews of nucleic acid chemistry can be found in the literature (204,205) and in chapter 15.

While not all drugs that are nucleic acid derivatives are oligomers, for those that are oligomeric, hydrolysis of the phosphodiester bond is one of the prominent degradation pathways. Such degradation leads to a break in the sugar-phosphate backbone as shown in Figure 144 for RNA strands. See Pogocki and Schöenich (205) for an in-depth discussion of this topic; in the case of phosphorothioate esters, see chapter 15.

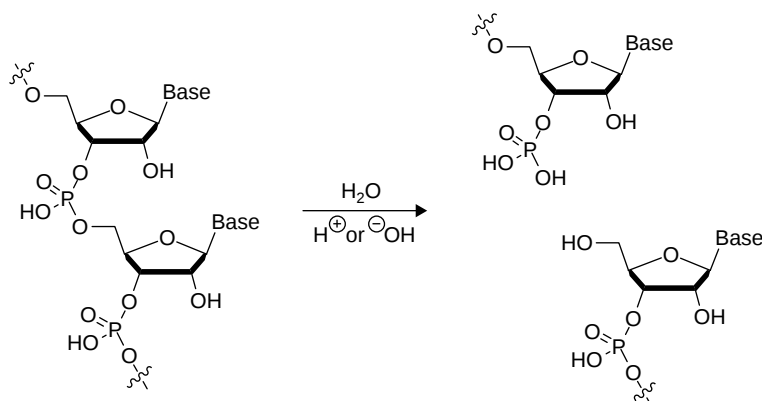


Figure 144 Hydrolysis of the phosphodiester backbond of an RNA strand.

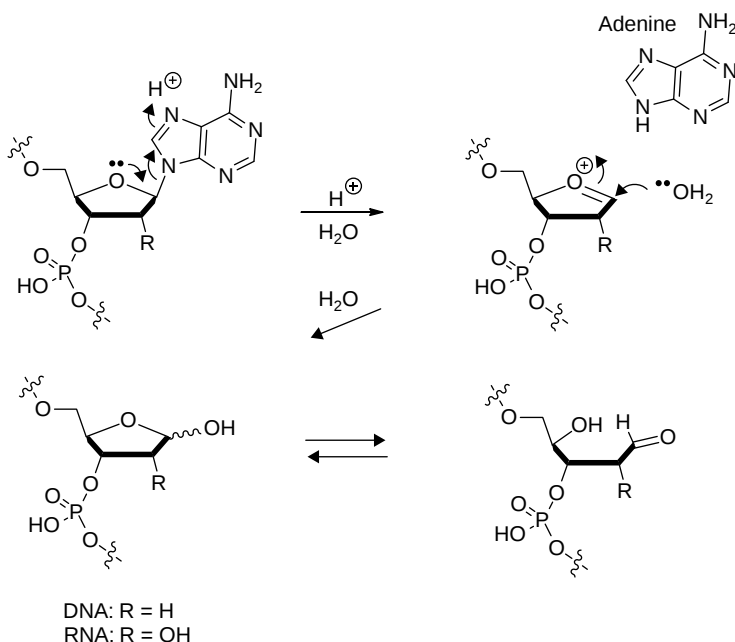


Figure 145 Acid-catalyzed depurination of a nucleic acid.

Depurination, or hydrolysis of the *N*-glycosidic bond, occurs for both RNA and DNA strands (oligomers /polymers) or analogs (monomers). RNA is much less prone to depurination than DNA as a result of the inductive effect of the 2'-OH group (206). Acid-catalyzed depurination is illustrated in Figure 145.

When the C2' R-group is strongly electron withdrawing, the nucleoside/tide can be significantly stabilized to depurination. Such is the case for gemcitabine (2'-deoxy-2',2'-difluorocytidine), a β -difluoronucleoside. The aqueous degradation of gemcitabine under acidic, mildly acidic (pH 3.2), and basic conditions has been studied and the degradation chemistry is shown in Figures 146 and 147 (207). As shown in the figures, depurination does not occur to a measurable extent; rather, nucleophilic attack of the cytidine at C6 (by either water or intramolecular attack by the 5'-hydroxyl) occurs first, leading eventually to deamination. Under basic conditions, a remarkable anomerization reaction occurs (shown in Fig. 147) illustrating the resistance of the difluoronucleoside to depurination.

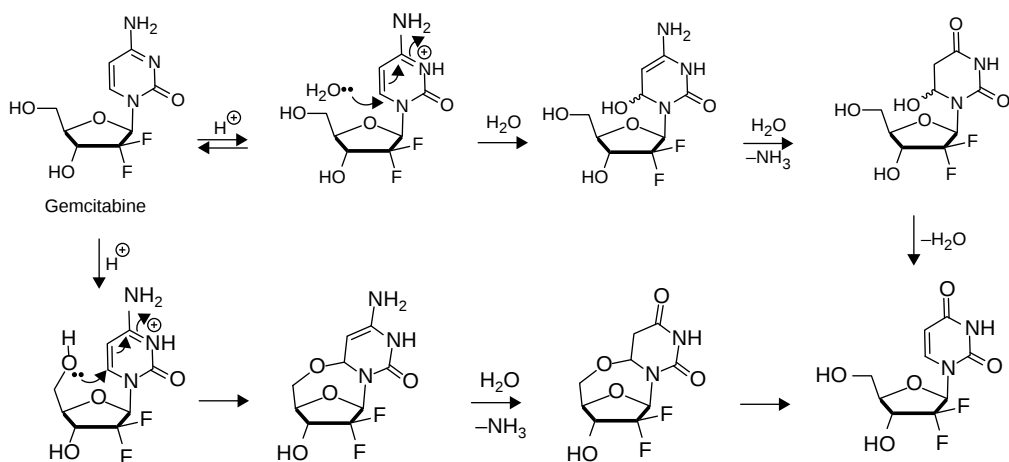


Figure 146 Proposed aqueous acidic degradation pathways of gemcitabine.

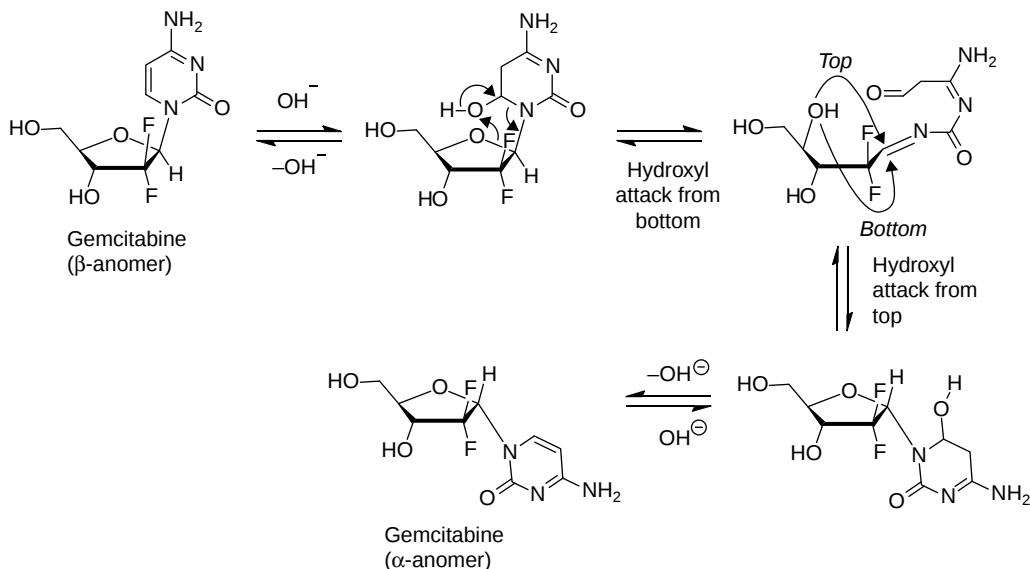


Figure 147 Base-catalyzed degradation of gemcitabine does not induce depurination, but rather reversible anomerization of the base from β to α .

Oxidative degradation of nucleic acids has been studied extensively. See the reviews by Pogocki and Schöenich (205) and by Waterman et al. (208) for excellent discussions of this topic. For a more detailed discussion, see chapter 15.

Amino Acids

Protein degradation commonly includes aggregation, deamidation, isomerization, racemization, disulfide bond exchange, hydrolysis, and oxidation (209). For an in-depth discussion of protein degradation, see chapter 14. Amino acid residues most likely to undergo degradation include asparagines (Asn), aspartic acid (Asp), methionine (Met), cysteine (Cys), glutamine (Gln), histidine (Hys), lysine (Lys), and serine (Ser). For liquid drug product formulations, the

major degradation pathways are hydrolysis, deamidation, and isomerization (210). For lyophilized powder and tablet formulations, the major degradation reactions are similar to the solution formulations, although removal of water through lyophilization results in reduced rates of hydrolysis. For additional discussion of protein stability in lyophilized formulations see chapters 13 and 14. In microsphere depot formulations, aggregation and oxidation are the main causes of peptide degradation.

Aggregation: Protein aggregation has two forms: noncovalent (involving the interaction of two or more denatured proteins) and covalent (e.g., disulfide bond formation and/or peptide condensation reactions).

Deamidation, Isomerization, and Racemization: These three reactions are common degradation pathways of proteins and peptides. These reactions are especially prevalent for peptides containing asparagine (Asn) and glutamine (Gln) residues. In the deamidation reaction (Fig. 148), the Asn or Gln amide side chains are hydrolyzed to form a carboxylic acid. During this deamidation process, an isomerization reaction can occur in which the peptide backbone is transferred from the α -carboxyl of the Asn or Gln to the side chain β or γ -carboxyl. Asn-Gly and Asn-Ser are most likely to deamidate. Deamidation is accelerated at alkaline pH conditions through a proposed cyclic imide intermediate. Under acidic conditions, direct deamidation occurs without the cyclic intermediate. In addition to the primary structure, the secondary and tertiary structures also influence the rate of deamidation. Denatured proteins allow increased conformational mobility to form the cyclic intermediate.

These reactions have been extensively described in the literature (211,212,213,214,215). It has been shown that at low pH (e.g., pH <2), although the cyclic imide does form, the deamidation occurs primarily via direct hydrolysis. When the pH is >5, however, the deamidation occurs exclusively via the cyclic imide. The isoAsp product resulting from deamidation is always formed in a 2- to 4-fold excess compared to the Asp product, although the excess is reduced as the pH rises. The maximum stability toward deamidation is generally in the pH 3–4 range.

In a racemization reaction, the configuration about the α -carbon of the amino acid is inverted. For Asn and Gln amino acids, the racemization is facilitated by enolization of the succinimide, as shown in Figure 149.

Isomerization of aspartyl residues in peptides or proteins can occur via a succinimide intermediate (Fig. 148) (216). The isomerized aspartyl residue can be detected by peptide

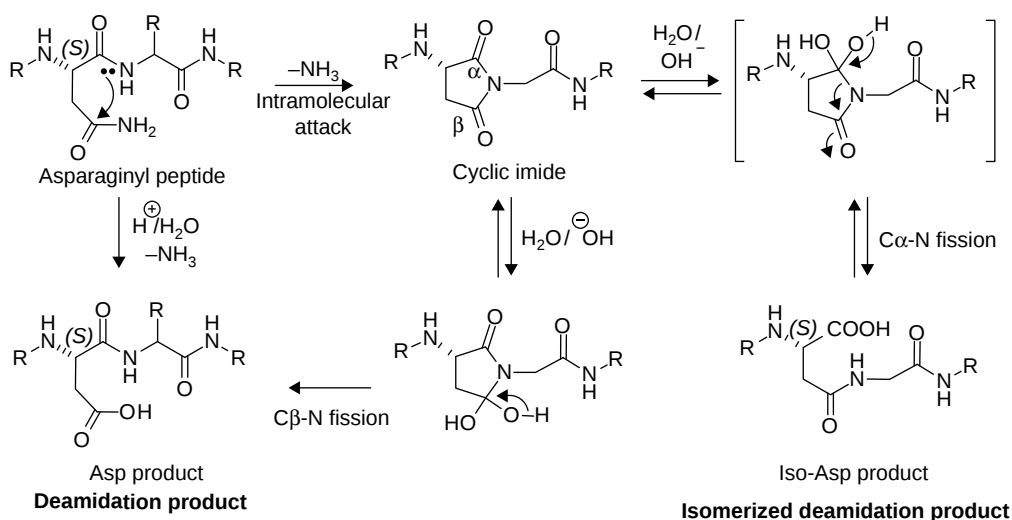


Figure 148 "Deamidation" reactions: asparagine to Asp and IsoAsp.

mapping, Edman sequencing, and selective methylation of the isoAsp peptide using carboxyl methyl transferase enzyme. Succinimide sites in proteins can be detected by basic hydroxylamine cleavage at the succinimide residue and subsequent *N*-terminal sequencing (217). Peptide bonds of aspartyl residues are cleaved under acidic conditions 100 times faster than other peptide bonds, with aspartyl-proline peptide bonds being most labile. Hydrolysis occurs at either the *N* or *C*-terminal peptide bonds adjacent to the aspartyl residue. Techniques such as SDS-PAGE and SDS-NGS CE are the most useful to detect peptide bond cleavage fragments.

Disulfide Bond Exchange: Disulfide linkages are important in determining protein tertiary structure. Disulfide bond formation and/or exchange may occur during metal-catalyzed oxidation of the cysteine residue. This may lead to protein aggregation due to the formation of intermolecular disulfide bonds. In addition to cysteine disulfide bond formation, cysteine is susceptible to oxidation (Fig. 150).

Hydrolysis: Peptide bonds of aspartic acid (Asp) residues are cleaved under dilute acidic conditions. Hydrolysis can take place at the *N*-terminal, the *C*-terminal or both terminal peptide bonds adjacent to the Asp residue.

Oxidation: The side chains of cysteine (Cys), histidine (His), methionine (Met), tryptophan (Trp), and tyrosine (Tyr) residues are susceptible to oxidation. Oxidation can result in loss of protein activity. Met is the most reactive residue, which oxidizes even with atmospheric oxygen to form *Met*-sulfoxide, is frequently observed in proteins (Fig. 151). Additional sources of oxidation include oxidizing agents (peroxides in excipients), metal-catalyzed oxidation and photo-oxidation. Oxidation can be detected analytically by reversed phase HPLC and HIC (hydrophobic interaction chromatography). Peptide mapping and mass spectrometry are

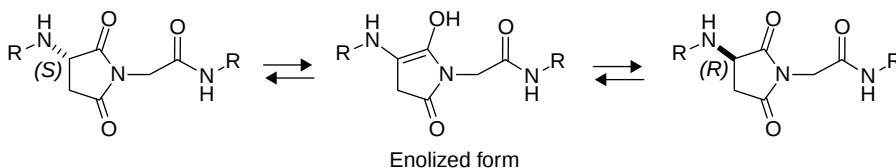


Figure 149 Racemization of an asparagine residue via a cyclic imide intermediate.

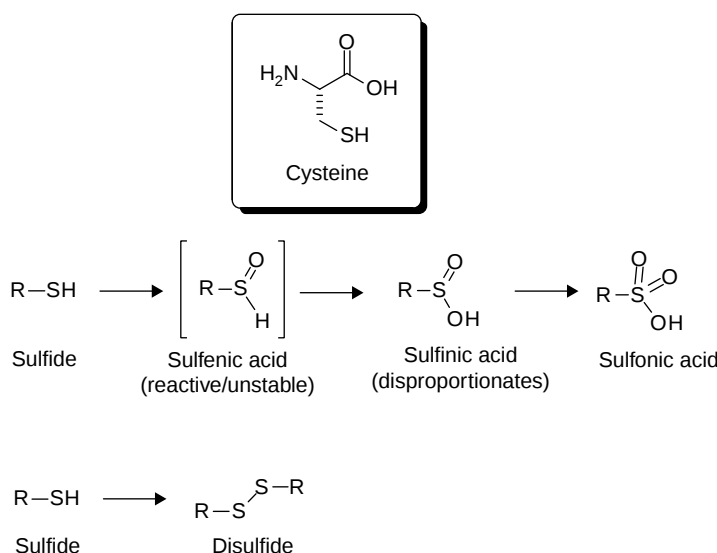


Figure 150 Cysteine oxidation and reactions.

useful for determination of oxidation sites. Methionine oxidation can occur by different mechanisms, via one- and two-electron oxidation processes. These processes can be evaluated using peroxides and HOCl or NaOCl (Figs. 151 and 152) (218,219).

The angiotension converting enzyme inhibitor captopril is a rare example of a drug containing a sulfur atom in the form of a thiol (Fig. 153). The degradation chemistry of this API mimics the protein cysteine by forming a disulfide dimer on long-term stability studies. Thiols have a tendency to form relatively stable ($RS\bullet$) radicals. These radicals can be formed by reacting with trace metals present in the API or by direct reaction with molecular oxygen when deprotonated to the anionic form. Once a thiol radical ($RS\bullet$) has been formed, a bond forming radical termination reaction (disulfide formation) is easily accomplished (220).

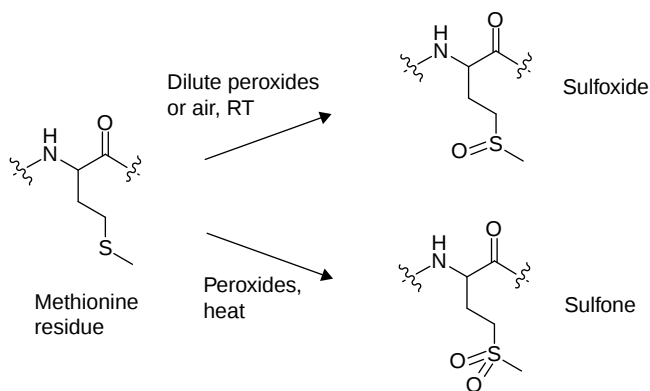


Figure 151 Methionine oxidation.

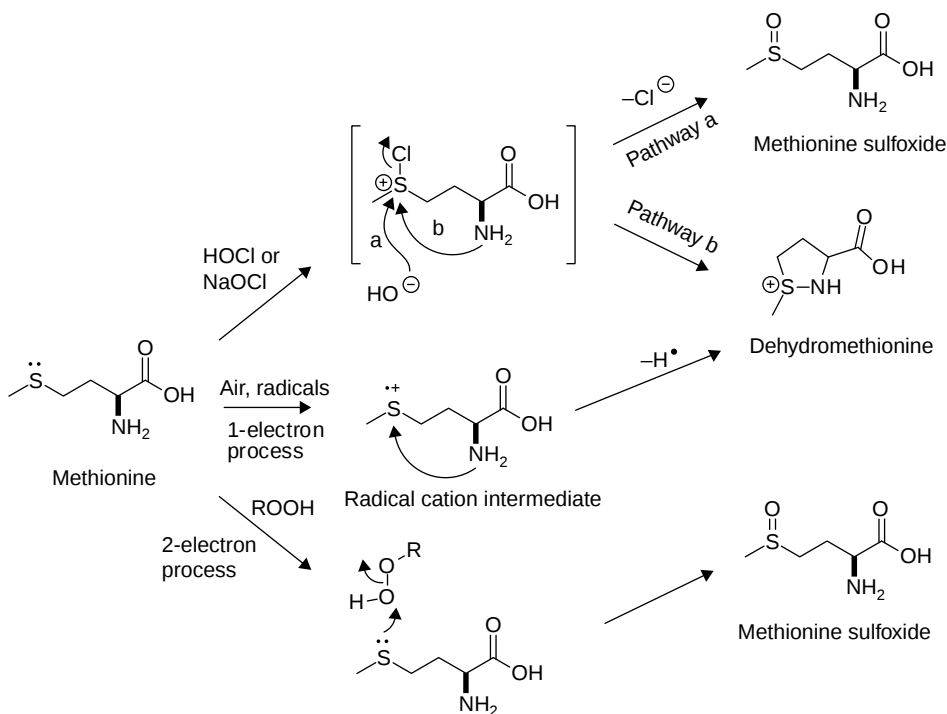


Figure 152 Potential methionine oxidation via one- and two-electron pathways.

Photodegradation: UV/Vis exposure can induce protein oxidation, aggregation, and backbone cleavage. For example, oxidation has been observed in the histidine residue of human growth hormone (hGH) exposed to photostability conditions (6.7×10^6 lux hour visible light exposure). The proposed oxidation sequence and products are shown in Figure 154.

Beta-Elimination: Beta-elimination of cysteine, serine, threonine, lysine, and phenylalanine residues proceed via a carbanion intermediate. This mechanism is influenced by metal ions and favored under basic conditions. A representative beta elimination is shown in Figure 155 from a disulfide linked residue (212).

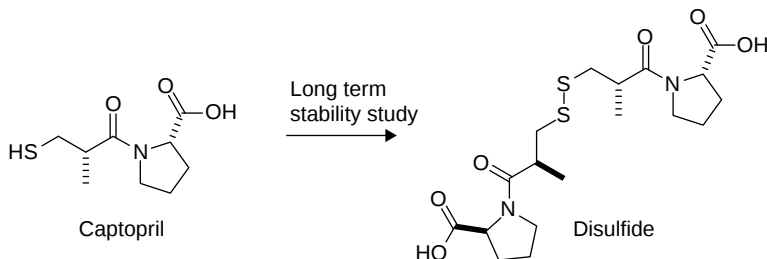


Figure 153 Captopril oxidation and disulfide bond formation.

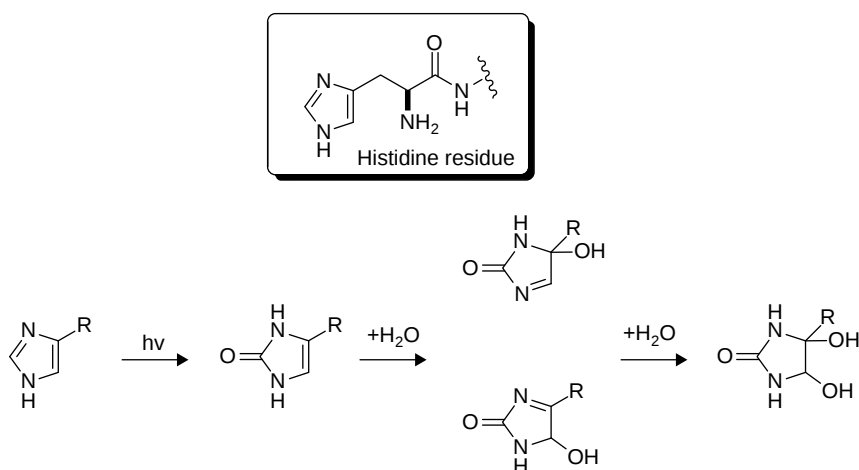


Figure 154 Histidine oxidation in human growth hormone.

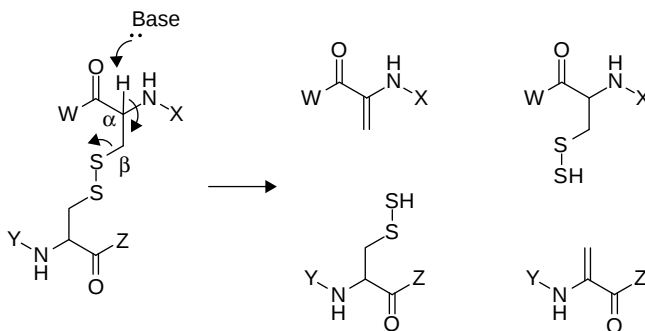
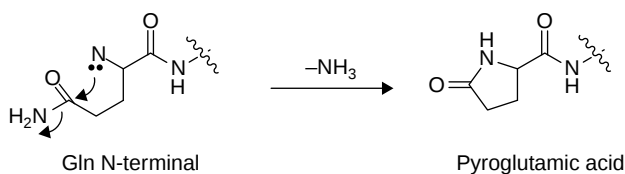
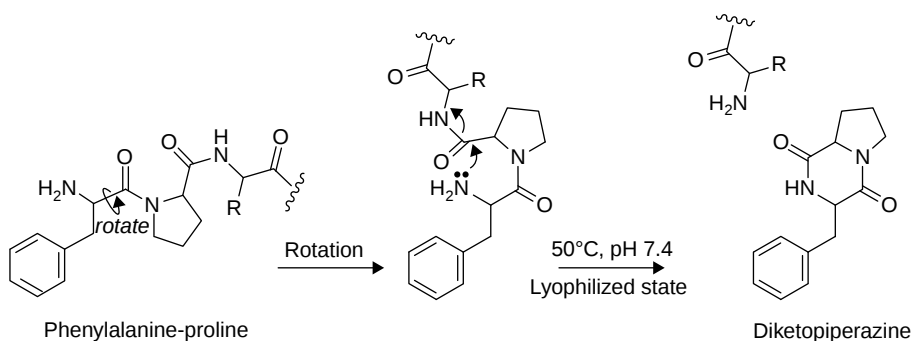


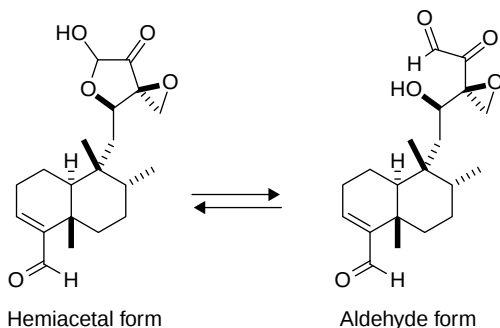
Figure 155 Beta-elimination from a cysteine residue in a protein.

**Figure 156** Pyroglutamic acid formation.**Figure 157** *N*-Terminal diketopiperazine formation.

Other degradation mechanisms: Additional degradation reactions include *N*-terminal degradation to form pyroglutamic acid (Fig. 156) (221) and *N*-terminal degradation to form a diketopiperazine (Fig. 157) (222).

Covalent Reactions of Pharmaceuticals with Buffers

While buffering systems are ideally assumed to be unreactive with pharmaceuticals, buffers are known to enhance certain reactions that are sensitive to acid/base catalysis. There are documented cases of covalent reactions with reagents/solvents and with APIs. For example, bicarbonate has been shown to react reversibly with amines, as demonstrated in the case of meropenem [discussed earlier in this chapter (Fig. 60)]. TRIS [tris(hydroxymethyl)aminomethane] has been shown to react with aldehydes (223,224). An example of covalent reaction with buffers is seen in the case of clerocidin, which has been shown to react with TRIS and with phosphate (225,226). Clerocidin is a complex microbial terpenoid characterized by the presence of several electrophilic groups—a strained epoxy ring, an α,β -unsaturated aldehyde, and a second aldehyde functionality that is α to a ketone and in equilibrium with a cyclic hemiacetal form (Fig. 158). In the presence of phosphate buffer (50 mM, pH 7.4, 37°C), the phosphate anion

**Figure 158** Structure of the two forms of clerocidin.

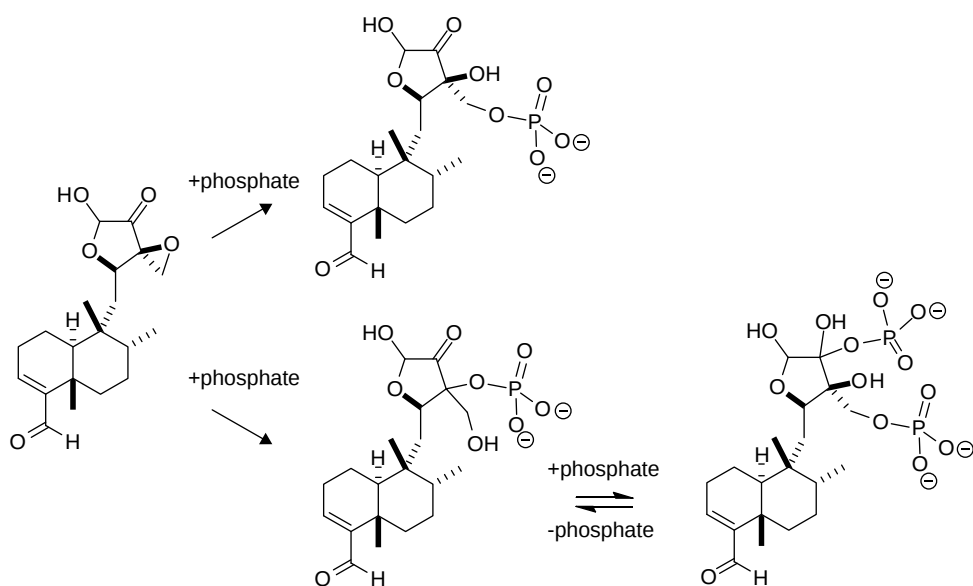


Figure 159 Reaction of clerocidin with phosphate buffer (pH 7.4).

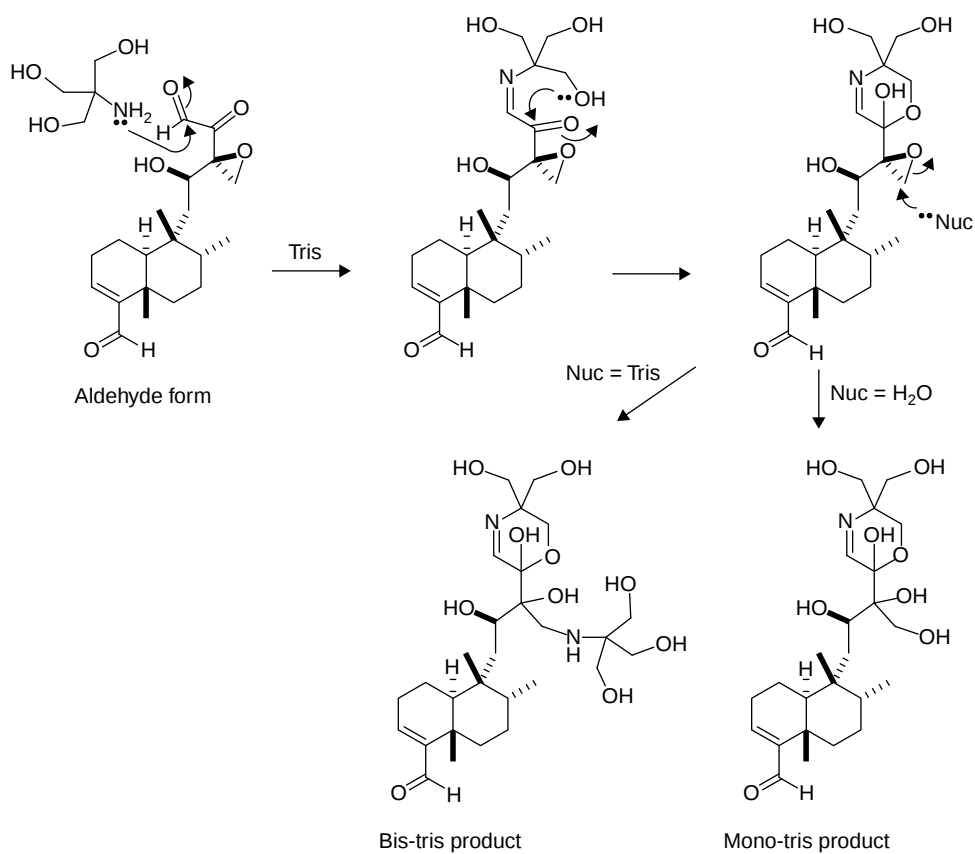


Figure 160 Reaction of clerocidin with Tris-buffer (pH 7.4).

attacks the epoxy ring of clerocidin to form two different mono-phosphate adducts (Fig. 159). A second molecule of phosphate can nucleophilically attack the electrophilic ketone to form a relatively unstable bis-adduct. In the presence of TRIS buffer (50 mM, pH 7.4, 37°C), the aldehydic carbon reacts with the nitrogen of TRIS to form an imine, and an intramolecular cyclization reaction then occurs from nucleophilic attack of one of the hydroxyls of TRIS on the ketone of clerocidin to form a hemiacetal (Fig. 160). Opening of the epoxy ring via either attack of water or attack of a second molecule of TRIS results in either a mono-TRIS or a bis-TRIS adduct.

Atropisomers

Atropisomers are conformers that result from hindered rotation about a single bond due to high steric or electronic constraints such that the conformers interconvert slowly enough that they can be isolated or detected spectroscopically (e.g., by proton NMR or via HPLC as separate peaks in a chromatogram). Atropisomers are identical in terms of bond-bond connections, MW, and chemical formula; however, atropisomers can be geometrical isomers, diastereoisomers, or enantiomers, which can all in principle be thermally equilibrated. Atropisomers may interact differently with an enzyme or receptor, which means that the API and the isomer may not be identical with respect to efficacy. Two notable examples of atropisomerism in pharmaceuticals are the drugs vancomycin (227) (Fig. 161) and oritavancin (228) (Fig. 162). In the case of vancomycin, and in the case of vancomycin, an intramolecular asparagines to isoasparagine rearrangement occurs via a succinimide intermediate, as shown in the figure. This rearrangement results in the expansion of the ring system by one carbon, thereby allowing the chlorophenyl ring to “rotate through” the macrocyclic ring system, resulting in an equilibrium

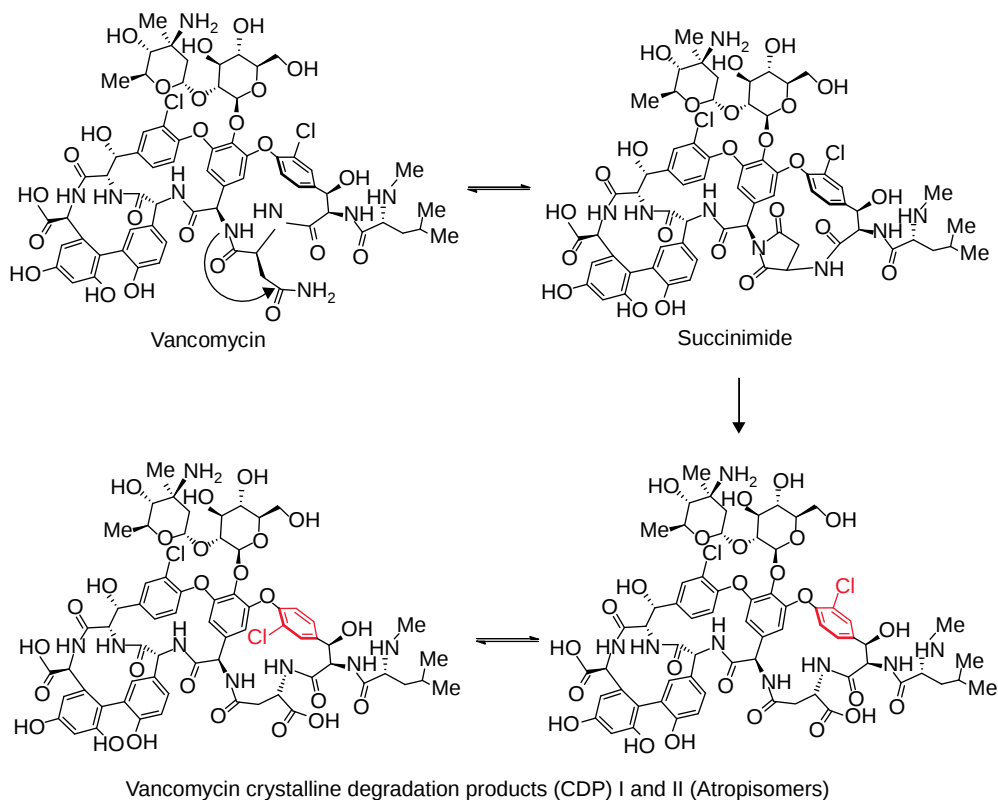


Figure 161 Degradation of vancomycin leading to equilibrium mixture of CDP-I and CDP-II atropisomers.

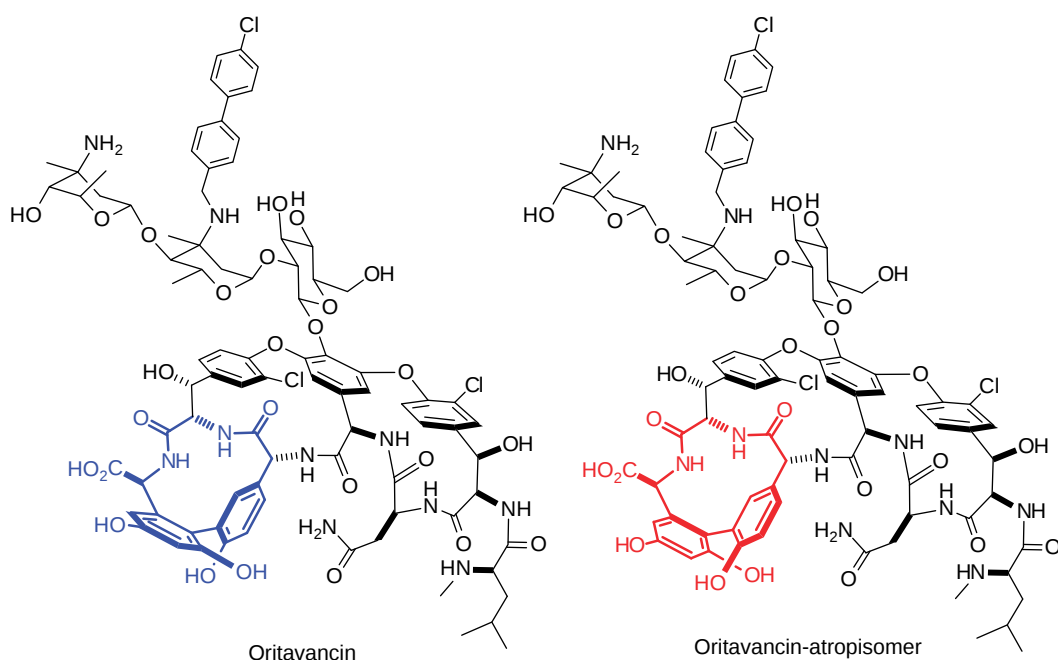


Figure 162 Oritavancin atropisomer.

mixture of atropisomers named crystalline degradation product (CDP)-I and CDP-II. In the case of oritavancin, a rotational twist of the bottom left hand portion of the molecule gives a different hindered AB-biaryl conformation to the compound. The differences between these isomers can be characterized by HPLC and NMR spectroscopy. A more thorough discussion of atropisomerism in drug discovery and the implications, both regulatory and otherwise, has been recently published (229).

CONCLUSION

We have attempted to document many of the major degradation pathways available to small molecule pharmaceuticals, based both on organic chemistry principles and on drug degradation examples available in the public literature. It is hoped that this chapter will serve as a useful resource for scientists around the world that are involved in developing an understanding of the stability and degradation of drugs, either being developed or already on the market. While we have tried to focus on the most common, predictable degradation pathways in drug degradation, which follow well-established organic chemistry “rules,” it is important to remember that degradation chemistry can be complex and can result in unusual or unexpected products. As the field of degradation chemistry matures, it is expected that many of these surprising pathways will be elucidated and new patterns and “rules” will emerge. The authors encourage continuing publication of drug degradation chemistry, enabling both the development of this field of chemistry as well as providing new knowledge that can help to speed innovative and safe medicines to the patient.

Acknowledgment

The authors acknowledge Patrick J. Jansen for review of the chapter content and mechanistic suggestions.

REFERENCES

1. Smith MB, March J. *Advanced Organic Chemistry. Reactions, Mechanisms, and Structure*. 5th edn. New York: John Wiley and Sons, 2001: 903.
2. (a) Stewart PJ, Tucker IG. Prediction of Drug Stability-Part 1: Mechanisms of drug degradation and basic rate laws. *Aust J Hosp Pharm* 1984; 14: 165–70. (b) Stewart PJ, Tucker IG. Prediction of Drug Stability-Part 2: Hydrolysis. *Aust J Hosp Pharm* 1985; 15: 11–16. (c) Stewart PJ, Tucker IG. Prediction of Drug Stability-Part 3: Oxidation. *Aust J Hosp Pharm* 1985; 15: 111–18. Stewart PJ, Tucker IG. Prediction of Drug Stability-Part 4: Isomerisation. *Aust J Hosp Pharm* 1985; 15: 181–8.
3. Reynolds DW, Facchine KL, Mullaney JF et al. Available guidance and best practices for conducting forced degradation studies. *Pharma Technol* 2002; 26.
4. Waterman KC, Adami RC. Accelerated aging: Prediction of chemical stability of pharmaceuticals. *Int J Pharm* 2005; 293: 101–25.
5. Waterman KC, Carella AJ, Gumkowski MJ et al. Improved protocol and data analysis for accelerated shelf-life estimation of solid dosage forms. *Pharm Res* 2007; 24: 780–90.
6. At the time of the search, there were 320 parent drugs and 1150 unique degradation products in the Pharma Drug Degradation Database.
7. Baertschi SW. Patterns and pathways: using chemistry to guide the characterization of degradation products. Presented at the Conference on Small Molecule Science, Boston, MA, August, 2009.
8. The Chemistry of Functional Groups [*Series Editor: Zvi Rappoport, The Hebrew University, Jerusalem, Israel*].
9. Shah RP, Kumar V, Singh S. Liquid chromatography/mass spectrometric studies on atorvastatin and its stress degradation products. *Rapid Commun Mass Spectrom* 2008; 22: 613.
10. March J. *Advanced Organic Chemistry: Reactions, Mechanisms, and Structure*, 3rd edn. New York: John Wiley and Sons, 1985: 334–8.
11. Mabey W, Mill T. Critical review of the hydrolysis of organic compounds in water under environmental conditions. *J Phys Chem Data* 1978; 7(2): 383–415.
12. Mabey W, Mill T. Hydrolysis of organic compounds. In: O. Hutzinger ed. *The Handbook of Environmental Chemistry*. Vol 2/Part D. Berlin: Springer-Verlag, 1988.
13. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol 8. New York: Academic, 1979: 30.
14. Brittain HG, ed. *Analytical Profiles of Drug Substances and Excipients*. Vol. 21. San Diego: Academic, 1992: 151.
15. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 5. New York: Academic, 1976: 545.
16. Brittain HG, ed. *Analytical Profiles of Drug Substances and Excipients*. Vol. 21. San Diego, CA: Academic, 1992: 280.
17. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 20. New York: Academic, 1991: 231.
18. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 3. New York: Academic, 1974: 39.
19. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 4. New York: Academic, 1975: 68.
20. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 13. New York: Academic, 1984: 229.
21. Florey K, ed. *Analytical Profiles of Drug Substances*, Vol. 14. New York: Academic, 1985: 227.
22. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 4. New York: Academic, 1989: 23.
23. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol 20. New York: Academic, 1991: 716.
24. Brittain HG, ed. *Analytical Profiles of Drug Substances and Excipients*. Vol 28. San Diego. Academic, 2001: 155.
25. Waterman KC, Adami RC, Alsante KM et al. Hydrolysis in pharmaceutical formulations. *Pharm Dev Technol* 2002; 7: 113–46.
26. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 14. New York: Academic, 1985: 227.
27. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 14. New York: Academic, 1985: 539.
28. For specific examples of articles dealing with β -lactam degradation chemistry see (a) Baertschi SW, Dorman DE, Occolowitz JL, et al. Isolation and characterization of degradation products arising from aqueous degradation of Cefaclor. *J Pharm Sci* 1997; 86: 526–39. (b) Dorman DE, Lorenz LJ, Occolowitz JL, et al. Isolation and structure elucidation of the major degradation products of Cefaclor in the solid state. *J Pharm Sci* 1997; 86: 540–9. (c) Skibic M, Taylor KW, Occolowitz JL, et al. Aqueous acidic degradation of the Carbacephalosporin Loracarbef. *J Pharm Sci* 1993; 82: 1010–17. (d) Bontchev PR, Papazova P. Hydrolysis of cephalosporins in strongly acidic medium. *Pharmazie* 1978; 33: 346–8. (e) Fuentes-Robinson VA, Jeffries TM, Branch SK. Degradation pathways of ampicillin in alkaline solutions. *J Pharm Pharmacol* 1997; 49: 843–51.
29. For general information on the chemistry of β -lactams see Flynn, EH ed. *Cephalosporins and Penicillins: Chemistry and Biology*. New York: Academic, 1972.

30. Waxman DJ, Strominger JL. Penicillin-binding proteins and the mechanism of action of beta-lactam antibiotics. *Ann Rev Biochem* 1983; 52: 825–69.
31. Page MI. The mechanisms of reactions of beta-lactam antibiotics. *Adv Phys Organic Chem* 1987; 23: 165–270.
32. Smith H, Marshall AC. Polymers formed by some β -lactam antibiotics. *Nature* 1971; 232: 45–6.
33. Larsen C, Bundaard, H. Polymerization of penicillins. *J Chrom* 1978; 147: 143–50.
34. Baertschi SW, Boyd DB, Cantrell AS et al. Inhibition of HIV-1 reverse transcriptase by degradation products of Ceftazidime. *Antiviral Chem Chemotherapy* 1997; 8: 353–62.
35. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 7. New York: Academic, 1978: 35.
36. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 1. New York: Academic, 1972: 263.
37. Kirby AJ, Lloyd GJ. Structure and efficiency in intramolecular and enzymic catalysis: intramolecular general base catalysis. Hydrolysis of monoaryl malonates. *J Chem Soc, Perkin Trans 2*, 1976: 1753–61.
38. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 5. New York: Academic, 1976: 163.
39. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 7. New York: Academic, 1978: 377.
40. March J. *Advanced Organic Chemistry. Reactions, Mechanisms, and Structure*. 3rd edn, New York: John Wiley and Sons, 1985: 229–30.
41. March J. *Advanced Organic Chemistry. Reactions, Mechanisms, and Structure*, 3rd edn.. New York: John Wiley and Sons, 1985: 562–5.
42. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 13. New York: Academic, 1984: 319.
43. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 14. New York: Academic, 1985: 513.
44. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 20. New York: Academic, 1991: 588.
45. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 20. New York: Academic, 1991: 717.
46. Brittain HG, ed. *Analytical Profiles of Drug Substances and Excipients*, Vol. 24. San Diego: Academic, 1996: 433.
47. Kochling JD, Miao H, Young CR et al. Understanding the degradation pathway of a poorly water-soluble drug formulated in PEG-400. *J Pharm Biomed Anal* 2007; 11: 1638–46.
48. Lowry, TH, Richardson, KS. Reactions of carbonyl compounds. In: *Mechanism and Theory in Organic Chemistry*, 2nd edn. New York: Harper and Row, 1981: 596–8.
49. (a) Wirth DD, Baertschi SW, Johnson, RA, et al. Maillard reaction of lactose and fluoxetine hydrochloride, a secondary amine. *J Pharm Sci* 1998; 87: 31–9. (b) Maillard LCC. *R Acad Sci Ser 2* 1912; 154: 66. (c) Maillard LCC. *R Acad Sci Ser 2* 1912; 155: 1554–6. (d) Ellis GP. The Maillard reaction. *Adv Carbohydr Chem* 1959; 14: 63–134. (e) Hodge JE. Dehydrated foods: Chemistry of browning reactions in model systems. *J Agric Food Chem* 1953; 1: 928–43.
50. March J. *Advanced Organic Chemistry. Reactions, Mechanisms, and Structure*, 3rd edn. New York: John Wiley and Sons, 1985: 829–31.
51. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 9. New York: Academic, 1980: 357.
52. Albini A, Fasani E. *Drugs: Photochemistry and Photostability. An Overview and Practical Problems*. Cambridge, UK: The Royal Society of Chemistry, 1998: 2.
53. Florey, K, ed. *Analytical Profiles of Drug Substances*. Vol. 1. New York: Academic, 1972: 411.
54. Payne GB, Deming PH, Williams PH. Reactions of hydrogen peroxide: VII. Alkali-catalyzed epoxidation and oxidation using nitrile as co-reactant. *J Org Chem* 1961; 26: 659–63.
55. Laus G. Kinetics of acetonitrile-assisted oxidation of tertiary amines by hydrogen peroxide. *J Chem Soc, Perkins Trans*. 2001; 2: 864–8.
56. Lu J, Lau C, Morizono M, Ohta K, Kai M. A Chemiluminescence reaction between hydrogen peroxide and acetonitrile and its applications. *Anal Chem* 2001; 73: 5979–83.
57. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 13. New York: Academic, 1984: 164.
58. Riesz P, Kondo T, Carmichael AJ. Sonochemistry of acetone and acetonitrile in aqueous solutions. A spin-trapping study. *Free Rad Res Comm* 1993; 19: S45–53.
59. Karran G, Legge M. Non-enzymatic formation of formaldehyde in mouse oocyte freezing mixtures. *Human Reproduction* 1996; 11(12): 2681–6.
60. Skibic MJ, King LA, Khan M et al. Artifactual formylation of duloxetine hydrochloride by acetonitrile in the presence of titanium dioxide and ultrasonication: implications for HPLC method development. *J Pharm Biomed Anal* 2010; 53: 432–9.
61. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 7. New York: Academic, 1978: 165.
62. Qin X-Z, Ip DP, Chang KH-C44 et al. Pharmaceutical application of LC-MS. 1-Characterization of a famotidine degradate in a package screening study by LC-APCI MS. *J Pharm Biomed Anal* 1994; 12: 221–33.

63. Francolic JD, Lehr GJ, Barry TL, Petzinger G. Isolation of a 2:1 hydrochlorothiazide-formaldehyde adduct impurity in hydrochlorothiazide drug substance by preparative chromatography and characterization by electrospray ionization LC-MS. *J Pharm Biomed Anal* 2001; 26: 651–63.
64. Waterman K, Arikpo WB, Fergione MB et al. N-Methylation and N-formylation of a secondary amine drug (Varenicline) in an osmotic tablet. *J Pharm Sci* 2008; 97: 1499.
65. Fujita M, Ueda T, Handa T. Generation of formaldehyde by pharmaceutical excipients and its absorption by meglumine. *Chem Pharm Bull* 2009; 57: 1096–9.
66. Schick M J, ed. *Nonionic Surfactants Physical Chemistry. Stability of the Polyoxyethylene Chain*, chap. 18, New York: Marcel Dekker, 1987: 1011. ISBN 0-8247-7530-9.
67. (a) Hartauer K, Arbuthnot G, Baertschi SW et al. Influence of peroxide impurities in povidone and crospovidone on the tablet stability of raloxifene hydrochloride. Identification and control of an oxidative degradation product. *Pharmaceutical Development and Technology* 2000; 5: 303–19. (b) Vermeire A, Remon JP. Stability and compatibility of morphine. *Int J Pharmaceutics* 1999; 187: 17–51. (c) Qin X, Frech P. Liquid chromatography/mass spectrometry (LC/MS) identification of photooxidative degradates of crystalline and amorphous MK-912. *J Pharm Sci* 2001; 90: 833–44.
68. March J. *Advanced Organic Chemistry. Reactions, Mechanisms, and Structure*, 3rd edn. New York: John Wiley and Sons, 1985: 909.
69. Zhao ZZ, Qin X-Z, Wu A, Yuan Y. Novel rearrangements of an N-oxide degradate formed from oxidation of a morpholine acetal substance P antagonist. *J Pharm Sci* 2004; 93: 1957–61.
70. Benigni R, Bossa C. Structure alerts for carcinogenicity, and the Salmonella assay system: a novel insight through the chemical relational databases technology. *Mutation Res* 2008; 659, 248–61.
71. Joule JA, Mills K, Smith GF. *Heterocyclic Chemistry*, 3rd edn. Cheltenham, UK: Stanley Thornes (Publishers) Ltd., 1998: 101.
72. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 12. New York: Academic, 1983: 122.
73. (a) Brittain HG, ed. *Analytical Profiles of Drug Substances and Excipients*. Vol. 21. San Diego: Academic, 1992: 151. (b) Brittain, H. G. (ed.) *Analytical Profiles of Drug Substances and Excipients*. Vol. 28. San Diego: Academic Press 2001: 111.
74. Brittain HG, ed. *Analytical Profiles of Drug Substances and Excipients*. Vol 26, San Diego: Academic, 1999: 86.
75. Brittain HG, ed. *Analytical Profiles of Drug Substances and Excipients*. Vol. 26. San Diego: Academic, 1999: 312.
76. Brittain HG, ed. *Analytical Profiles of Drug Substances and Excipients*. Vol. 22. San Diego: Academic, 1993: 86.
77. Chafetz L, Hong W-H, Tsilifonis DC, Taylor AK, Philip J. Decrease in the rate of capsule dissolution due to formaldehyde from polysorbate 80 autoxidation. *J Pharm Sci* 1984; 73: 1186–7.
78. Bindra DS, Williams TD, Stella VJ. Degradation of O⁶-benzylguanine in aqueous polyethylene glycol 400 (PEG 400) solutions: concerns with formaldehyde in PEG 400. *Pharm Res* 1994; 11: 1060–4.
79. (a) Houminer Y, Hoz S. Formation of formaldehyde in the thermal decomposition of D-glucose labelled with ¹⁴C at various positions. *Israel J Chem* 1979; 8: 97–98. (b) Ferrier RJ, Severn WB, Furneaux RH, Miller IJ. Isotope studies of the transfer of the carbon atoms of carbohydrate derivatives into aromatic compounds (especially xanthene) under degradation conditions. *Carbohydr Res* 1992; 237: 87–94. (c) Ponder GR, Richards GN. Pyrolysis of inulin, glucose, and fructose. *Carbohydr Res* 1993; 244: 341–59.
80. (a) Walker JF. Formaldehyde, 2nd edn, American Chemical Society Monograph Series. Baltimore, MD: Waverly Press, 1953: 311–17. (b) Kirk-Othmer. *Encyclopedia of Chemical Technology*, 3rd edn. Vol. 11. New York: Wiley, 1980: 911.
81. Marks EM, Toutellote D, Andux A. The phenomenon of gelatin insolubility. *Food Technol* 1968; 22: 1433.
82. (a) Ofner CM III, Zhang Y-E, Jobeck VC, Bowman BJ. Cross-linking studies in gelatin capsules treated with formaldehyde and in capsules exposed to elevated temperature and humidity. *J Pharm Sci* 2001; 90: 79–88. (b) Digenis GA, Gold TB, Shah VP. Cross-linking of gelatin capsules and its relevance to their in vitro-in vivo performance. *J Pharm Sci* 1994; 83: 915–21. (c) Desai DS, Rubitski BA, Varia SA, Huang MH. Effect of formaldehyde formation on dissolution stability of hydrochlorothiazide bead formulations. *Int J Pharm* 1994; 107: 141–7.
83. Desai DS, Rubitski BA, Bergum JS, Varia SA. Effects of different types of lactose and disintegrant on dissolution stability of hydrochlorothiazide capsule formulations. *Int J Pharm* 1994; 110: 257–65.
84. Stephenson SA, Bryant DK, Thomson CM, Walsgrove TC, Webb ML. An analytical study of the interaction of low levels of formaldehyde with active pharmaceuticals. *J Pharm Pharmacol* 1998; 50: 122.

85. (a) Belongia EA, Hedlberg CW, Gleich GJ et al. An investigation of the cause of the eosinophilia-myalgia syndrome associated with tryptophan use. *N Engl J Med* 1990; 323: 357–65. (b) Smith MJ, Mazzola EP, Farrell TJ et al. 1,1'-Ethylidenebis(L-tryptophan), structure determination of contaminant "97" - implicated in the eosinophilia-myalgia syndrome (EMS). *Tetrahedron Lett* 1991; 32: 991–4. (c) Toyoda M, Saito Y, Uchiyama M et al. Formation of a 3-phenylamino)alanine contaminant in EMS-associated L-tryptophan. *Biosci Biotech Biochem* 1994; 58: 1318–20.
86. Mayeno AN, Lin F, Foote CS et al. Characterization of "Peak E", a novel amino acid associated with eosinophilia-myalgia syndrome. *Science* 1990; 250: 1707–08.
87. Hill RH, Jr., Caudill SP, Philen RM et al. Contaminants in L-tryptophan associated with eosinophilia myalgia syndrome. *Arch Environ Contam Toxicol* 1993; 25: 134–2.
88. Castello RA, Mattocks AM. Discoloration of tablets containing amines and lactose. *J Pharm Sci* 1962; 51: 106–8.
89. (a) Yaylayan VA, Huyghues-Despointes A. Chemistry of Amadori rearrangement products: analysis, synthesis, kinetics, reactions, and spectroscopic properties. *Cri Rev Food Sci Nutr* 1994; 34: 321–69. (b) Colaco C, Collett M, Roser B. Pharmaceutical formulation instability and the Maillard Reaction. *Chem Oggi* 1996; 14: 32–7.
90. Nursten HE. *The Maillard Reaction: Chemistry, Biology, and Implications*. London, UK : The Royal Society of Chemistry, 2005.
91. Harmon PA, Yin W, Bowen WE, Tyrrell RJ, Reed RA. Liquid chromatography-mass spectrometry and proton nuclear magnetic resonance characterization of trace level condensation products formed between lactose and the amine-containing diuretic hydrochlorothiazide. *J Pharm Sci*, 2000; 89(7): 920–9.
92. Gokhale MW, Kearney WR, Kirsch LE. Glycosylation of Aromatic Amines: I. Characterization of Reaction Products and Kinetic Scheme. *AAPS Pharm Sci Tech*, 2009; 10: 317–28.
93. Hodge JE. The Amadori Rearrangement. *Adv Carbohydr Chem* 1955; 10: 169–205.
94. Hurley TR, Lovdahl MJ, Priebe SR, Tobias B. Synthesis and characterization of pregabalin lactose conjugate degradation products. Detecting, Identifying, and Quantitating Impurities, Princeton, NJ : Institute for International Research, May 20–21, 2002.
95. Tjan SB, van den Ouweland GAM. PMR investigation into the structure of some N-substituted 1-amino-1-deoxy-D-fructoses (Amadori rearrangement products). *Tetrahedron* 1974; 30: 2891–97.
96. Williamson ML, Jansen PJ, Montgomery R et al., in press.
97. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 20. New York: Academic, 1991: 395.
98. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 20. New York: Academic, 1991: 557.
99. Schildcrout SA, Risley DS, Kleeman RL. Drug-excipient interactions of seproxetine maleate hemihydrate: isothermal stress methods. *Drug Dev Ind Pharm* 1993; 19: 1113–30.
100. Baertschi SW, et al., Eli Lilly and Company, unpublished results.
101. Jansen PJ, Oren PL, Kemp CA, Maple SR, Baertschi SW. Characterization of impurities formed by interaction of duloxetine HCl with enteric polymers hydroxypropyl methylcellulose acetate succinate (HPMCAS) and hydroxypropyl methylcellulose phthalate (HPMCP). *J Pharm Sci* 1998; 87: 81–5.
102. Almarsson Ö, Kaufman MJ, Stong JD et al. Meropenem exists in equilibrium with a carbon dioxide adduct in bicarbonate solution. *J Pharm Sci* 1998; 87.
103. Alsante KM, Salisbury JJ, Hatajik TD, Snyder K D. Investigation of ICH photostability for purposeful degradation studies: Option 1 vs. Option II, *Pharmaceutical Photostability 01*. Research Triangle Park, NC, 19 July, 2001.
104. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 4. New York: Academic, 1975: 259.
105. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol.19. New York: Academic, 1990: 615.
106. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol.1. New York: Academic, 1972: 93.
107. Florey K, ed. *Analytical Profiles of Drug Substances*, Vol.14. New York: Academic, 1985: 148.
108. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol.3. New York: Academic, 1974: 323.
109. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol.4. New York: Academic, 1975: 109.
110. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol.6. New York: Academic, 1977: 73.
111. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol.4. New York: Academic, 1975: 259.
112. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol.5. New York: Academic, 1976: 417.
113. Carlin A, Gregory N, Simmons J. *J Pharm Biomed Anal* 1998; 17, 885–90.
114. Smith M B, March J. *Advanced Organic Chemistry. Reactions, Mechanisms, and Structure*, 5th edn. New York: John Wiley and Sons, 2001: 787.
115. Smith M B, March J. *Advanced Organic Chemistry. Reactions, Mechanisms, and Structure*, 5th edn. New York: John Wiley and Sons, 2001: 1178.
116. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 19. New York: Academic, 1990: 42.

117. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 18. New York: Academic, 1989: 245.
118. Albini A, Fasani E. *Drugs: Photochemistry and Photostability. An Overview and Practical Problems*. Cambridge, UK: The Royal Society of Chemistry, 1998: 21.
119. Smith MB, March J. *Advanced Organic Chemistry. Reactions, Mechanisms, and Structure*, 5th edn. New York: John Wiley and Sons, 2001: 576.
120. Elder DP, Delaney E, Teasdale A et al. The Utility of sulfonate salts in drug development. *J Pharm Sci* 2010; 2948–61.
121. Horspool WM. *CRC Handbook of Photochemistry and Photobiology*, chap. 6. Boca Raton: CRC Press, 1995: 758.
122. Brittain HG, ed. *Analytical Profiles of Drug Substances and Excipients*. Vol. 26. San Diego: Academic, 1999: 87.
123. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 6. New York: Academic, 1977: 539.
124. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 7. New York: Academic, 1978: 414.
125. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 10. New York: Academic, 1981: 345.
126. Hovorka SW, Schöneich C. Oxidative degradation of pharmaceuticals: theory, mechanisms, and inhibition. *J Pharm Sci* 2001; 90: 253–69.
127. Luo D, Smith SW, Anderson BD. Kinetics and mechanism of the reaction of cysteine and hydrogen peroxide in aqueous solution. *J Pharm Sci* 2005; 94: 304–16.
128. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 16. New York: Academic, 1987: 676.
129. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 9. New York: Academic, 1980: 138.
130. Florey K, ed. *Analytical Profiles of Drug Substances*, Vol.13. New York: Academic, 1984: 318.
131. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 2. New York: Academic, 1973: 256.
132. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 13. New York: Academic, 1984: 165.
133. Brittain HG, ed. *Analytical Profiles of Drug Substances and Excipients*. Vol. 21. San Diego: Academic, 1992: 375.
134. Florey K, ed. *Analytical Profiles of Drug Substances* Vol 19. New York: Academic, 1990: 421.
135. Brittain HG, ed. *Analytical Profiles of Drug Substances and Excipients*. Vol 21. San Diego: Academic, 1992: 409.
136. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol 6. New York: Academic, 1977: 163.
137. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 16. New York: Academic, 1987: 383.
138. Loudon GM. *Organic Chemistry*, 2nd edn. Menlo Park, CA: Benjamin/Cummings 1988: 319–67.
139. Smith MB, March J. *March's Advanced Organic Chemistry: Reactions, Mechanisms, and Structure*, Sixth Edition. Hoboken: John Wiley and Sons, 2007: 1478–86.
140. Brandl M, Wu X, Liu Y et al. Chemical reactivity of R0-26-9228, 1 α -fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol in aqueous solution. *J Pharm Sci* 2003; 92.
141. Bontchev PR, Papazova P. Hydrolysis of cephalosporins in strongly acidic medium. *Pharmazie* 1978; 33: 346–8.
142. Brittain HG, ed. *Analytical Profiles of Drug Substances and Excipients*. Vol. 21. San Diego: Academic, 1992: 299.
143. Martínez AG, Alvarez RM, Vilar ET et al. An improved modification of Ritter reaction. *Tet Lett* 1989; 30: 581–2.
144. Alsante KM, Hatajik TD, Lohr LL, Santafianos D, Sharp TR. Solving Impurity/Degradation Problems: Case Studies. In: Ahuja, S, Alsante, KM, eds. *Handbook of Isolation and Characterization of Impurities in Pharmaceuticals*, chap 14. Vol. 5; *Separation Sci Technol*. 2003: 361–400.
145. For a review see Mihailović and Čeković, "Oxidation and reduction of phenols", in *The chemistry of the hydroxyl group*, Part 1, Patai, S., ed., chap. 10, New York: Interscience Publishers, 1971.
146. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 7. New York: Academic, 1970: 215.
147. Albini A, Fasani E. *Drugs: Photochemistry and Photostability. An Overview and Practical Problems*. Cambridge, UK: The Royal Society of Chemistry, 1998: 23.
148. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 11. New York: Academic, 1982: 562.
149. March J. *Advanced Organic Chemistry. Reactions, Mechanisms, and Structure*, 4th edn. New York, John Wiley and Sons, 1992: 860.
150. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 13. New York: Academic, 1984: 289.
151. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 4. New York: Academic, 1975: 68.
152. Moore DE, Roberts-Thompson S, Zhen D, Duke CC. Photochemical studies on the anti-inflammatory drug diclofenac. *Photochem Photobiol* 1990; 52: 685–90.
153. Paillous N, Verrier M. Photolysis of amiodarone, an antiarrhythmic drug. *Photochem Photobiol* 1988; 47: 337–43.

154. Fasani E, Barberis Negra FF, Mella M, Monti S, Albini A. Photoinduced C-F bond cleavage in some fluorinated 7-amino-4-quinolone-3-carboxylic acids. *J Org Chem* 1999; 64: 5388–95.
155. Albini A, Fasani E. *Drugs: Photochemistry and Photostability. An Overview and Practical Problems.* Cambridge, UK: The Royal Society of Chemistry, 1998: 7.
156. Albini A, Fasani E. *Drugs: Photochemistry and Photostability. An Overview and Practical Problems.* Cambridge, UK: The Royal Society of Chemistry, 1998: 20.
157. Walling C. Some properties of radical reactions important in synthesis. *Tetrahedron* 1985; 41: 3887–90.
158. Gundermann KD, McCapra F. *Chemiluminescence in Organic Chemistry.* Berlin: Springer-Verlag 1987: 22–4.
159. Russell GA. Deuterium-isotope effects in the autoxidation of aralkyl hydrocarbons. mechanism of the interaction of peroxy radicals. *J Am Chem Soc* 1957; 79: 3871–77.
160. March J. *Advanced Organic Chemistry. Reactions, Mechanisms, and Structure*, 3rd edn. New York: John Wiley and Sons 1985: 164.
161. Florey K, ed. *Analytical Profiles of Drug Substances.* Vol. 14. New York: Academic, 1985: 59.
162. Waterman KC, Roy MC. Use of oxygen scavengers to stabilize solid pharmaceutical dosage forms: A case study. *Pharm Dev Tech* 2002; 7: 227–34.
163. Florey K, ed. *Analytical Profiles of Drug Substances.* Vol. 20. New York: Academic, 1991: 405.
164. Brittain HG, ed. *Analytical Profiles of Drug Substances and Excipients.* Vol. 21. San Diego: Academic, 1992: 163.
165. Brittain HG, ed. *Analytical Profiles of Drug Substances and Excipients* Vol. 27. San Diego: Academic, 2001: 293.
166. Smith MB, March J. *Advanced Organic Chemistry. Reactions, Mechanisms, and Structure*, 5th edn. New York: John Wiley and Sons, 2001: 1049.
167. Florey K, ed. *Analytical Profiles of Drug Substances* Vol. 7. New York: Academic, 1978: 125.
168. Florey K, ed. *Analytical Profiles of Drug Substances.* Vol. 17. New York: Academic, 1988: 177.
169. Florey K, ed. *Analytical Profiles of Drug Substances.* Vol. 18. New York: Academic, 1989: 553.
170. Brittain HG, ed. *Analytical Profiles of Drug Substances and Excipients.* Vol. 21. San Diego: Academic, 1992: 299.
171. Lowry TH, Richardson KS. *Mechanism and Theory in Organic Chemistry*, 3rd edn. New York: Harper & Row, 1987: 903.
172. Smith MB, March J. *Advanced Organic Chemistry. Reactions, Mechanisms, and Structure*, 5th edn. New York: John Wiley and Sons, 2001: 903.
173. Wang Y, Taylor J-S, Gross ML. Nuclease P1 digestion combined with tandem mass spectrometry for the structure determination of DNA photoproducts. *Chem Res Toxicol* 1999; 12: 1077–82.
174. Saitou M, Hieda K. Dithymine photodimers and photodecomposition products of thymidyl-ly-thymidine induced by ultraviolet radiation from 150 to 300 nm. *Radiat Res*, 1994; 140: 215–20.
175. Monroe BM. Rates of reaction of singlet oxygen with olefins. *J Phys Chem* 1978, 82, 15–8.
176. Lowry TH, Richardson KS. *Mechanism and Theory in Organic Chemistry*, 3rd edn. New York: Harper & Row, 1987: 1009.
177. Reddy M, Reddy V, Srinivas U, Reddy M, Rao V. Regioselective E(trans)-Z(cis) photoisomerization in naphthylidene derivatives. *Proc Indian Acad Sci (Chem Sci)*, 2002; 114: 603–9.
178. (a) Morliere P, Avice O, Melo T et al. A study of the photochemical properties of some cinnamate sunscreens by steady state and laser flash photolysis. *Photochem Photobiol* 1982; 36: 395–9. (b) Broadbent J, Martincigh B, Raynor M et al. Capillary supercritical fluid chromatography combined with atmospheric pressure chemical ionization mass spectrometry for the investigation of photoproduct formation in the sunscreen absorber 2-ethylhexyl-p-methoxycinnamate. *J Chrom* 1996; 732: 101–10.
179. Porter NA, Caldwell SE, Mills KA. Mechanisms of free radical oxidation of unsaturated lipids. *Lipids* 1995; 30: 277–89.
180. For review articles on mechanisms of autoxidation of polyunsaturated lipids see: (a) Porter NA, Caldwell SE, Mills KA. Mechanisms of free radical oxidation of unsaturated lipids. *Lipids* 1995; 30: 277–90. (b) Porter NA. Mechanisms for the autoxidation of polyunsaturated lipids. *Acc Chem Res* 1986; 19: 262–8. (c) Rouzer CA, Marnett LJ. Mechanism of free radical oxygenation of polyunsaturated fatty acids by cyclooxygenases. *Chem Rev* 2003; 103: 2239–304.
181. Morrow JD, Awad JA, Boss HJ, Blair IA, Roberts LJ II. Non-cyclooxygenase-derived prostanoids (F2-isoprostanes) are formed in situ on phospholipids. *Proc Nat Acad Sci* 1992; 89: 10721–25.
182. Morrow JD, Roberts LJ II. The isoprostanes: unique bioactive products of lipid peroxidation. *Prog Lipid Res* 1997; 36: 1–21.

183. Roberts LJ II, Montine TJ, Markesbury WR et al. Formation of isoprostane-like compounds (neuroprostanes) in vivo from docosahexaenoic acid. *J Biol Chem* 1998; 273: 13605–12.
184. (a) Nelson NA, Kelly RC, Johnson RA. Prostaglandins and the arachidonic acid cascade. *Chem. Eng News* 1982; 60: 30–44; (b) Granström E. The arachidonic acid cascade. The prostaglandins, thromboxanes and leukotrienes. *Inflammation* 1984; 8(1): S15–25.
185. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 4. New York: Academic, 1975: 399.
186. Brittain HG, ed. *Analytical Profiles of Drug Substances and Excipients*. Vol. 26. San Diego, CA: Academic, 1999: 87.
187. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 12. New York: Academic, 1983: 393.
188. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 9. New York: Academic, 1980: 411.
189. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 16. New York: Academic, 1987: 617.
190. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 20. New York: Academic, 1991: 588.
191. Feng W, Liu H, Chen G et al. Structural characterization of the oxidative degradation products of an antifungal agent SCH 56592 by LC–NMR and LC–MS. *J Pharm Biomed Anal* 2001; 25: 545–57.
192. Brittain HG, ed. *Analytical Profiles of Drug Substances and Excipients*. Vol. 22. San Diego: Academic, 1993: 254.
193. Florey K, ed. *Analytical Profiles of Drug Substances*. Vol. 18. New York: Academic, 1989: 141.
194. Pop E, Huang M-J, Brewster ME, Bodor N. On the mechanism of cephalosporin isomerization. *J Mol Struct (Theochem)* 1994; 315: 1–7.
195. Richter WF, Chong YH, Stella VJ. On the mechanism of isomerization of cephalosporin esters. *J Pharm Sci* 1990; 79: 185–6.
196. Baker MT, Gregerson MS, Martin SM, Buettner GR. Free radical and drug oxidation products in an intensive care unit sedative: propofol with sulfite. *Crit. Care Med.* 2003; 31: 787–92.
197. Florey K, ed. *Analytical Profiles of Drug Substances*, Vol. 8. New York: Academic Press, 1979: 383.
198. Garrett R, Grisham CM. *Biochemistry*, 4th edn, Florence, KY: Brooks/Cole Gengage Learning, Inc. 2010: 184.
199. Louden GM. *Organic Chemistry*, 2nd edn. Reading, MA: The Benjamin Cummings Publishing, 1988: 1210.
200. Juaristi E, Cuevas G. *The Anomeric Effect*. Boca Raton, FL: CRC Press, 1994.
201. Carey FA, Sunberg RJ. *Advanced Organic Chemistry, Part A: Structure and Mechanism*, 3rd edn. New York: Springer Science+Business Media Inc, 1990: 147–9.
202. Juaristi E, Cuevas G. Recent studies of the anomeric effect. *Tetrahedron* 1992; 48: 5019–87.
203. Brittain HG, ed. *Analytical Profiles of Drug Substances and Excipients*. Vol. 23. San Diego, CA: Academic, 1994: 305.
204. Miller PS. A brief guide to nucleic acid chemistry. *Bioconjugate Chem* 1990; 1: 187–91.
205. Pogocki D, Schöenich C. Chemical stability of nucleic acid-derived drugs. *J Pharm Sci* 2000; 89: 443–56, and references cited therein.
206. Lindahl T. Instability of the primary structure of DNA. *Nature* 1993; 362: 709–15.
207. (a) Anliker SL, McClure MS, Britton TC et al. Degradation chemistry of gemcitabine hydrochloride, a new antitumor agent. *J Pharm Sci* 1994; 83: 716–19. (b) Jansen PJ, Akers MJ, Amos RM et al. The degradation of the antitumor agent gemcitabine hydrochloride in an acidic aqueous solution at pH 3.2 and identification of degradation products. *J Pharm Sci* 2000; 89: 885–91.
208. Waterman KC, Adami RC, Alsante KA et al. Stabilization of pharmaceuticals to oxidative degradation. *Pharm Dev Tech* 2002; 7: 1–32.
209. Yu J. Intentionally degrading protein pharmaceuticals to validate stability-indicating analytical methods. *BioPharm.* 2000; 13: 46–50.
210. Niu C-H, Chiu Y-Y. FDA Perspective on peptide formulation and stability issues. *J Pharm Sci* 1998; 87 1331–4.
211. Geiger T, Clark S. Deamidation, isomerization, and racemization at asparaginyl and aspartyl residues in peptides. *J Biol Chem* 1987; 262: 785–94.
212. Capasso S, Kirby AJ, Salvadori S, Sica F, Zagari A. Kinetics and mechanism of the reversible isomerization of aspartic acid residues in tetrapeptides. *J Chem Soc Perkin Trans* 1992; 2: 437–42.
213. Capasso S, Mazarella L, Zagari A. Deamidation via cyclic imide of asparaginyl peptides: dependence on salts, buffers, and organic solvents. *Peptide Research* 1991; 4: 234–8.
214. Patel K, Borchardt RT. Chemical pathways of peptide degradation: II. Kinetics of deamidation of an asparaginyl residue in a model hexapeptide. *Pharm Res* 1990; 7: 703–11.

215. Xie M, Vander Velde D, Morton M, Borchardt RT, Schowen RL. pH-Induced change in the rate-determining step for the hydrolysis of the Asp/Asn-derived cyclic-imide intermediate in protein degradation. *J Am Chem Soc* 1996; 118: 8955–6.
216. For isomerization articles see: (a) Aswad DW. Stoichiometric methylation of porcine adrenocorticotropin by protein carboxyl methyltransferase requires deamidation of asparagine 25. Evidence for methylation at the alpha-carboxyl group of atypical L-isoaspartyl residues. *J Biol Chem* 1984; 259: 10714–21; (b) Johnson BA, Shirokawa J, Hancock W et al. Formation of isoaspartate at two distinct sites during in vitro aging of human growth hormone. *J Biol Chem* 1989; 264: 14262–71.
217. Kwong M, Harris R. Identification of succinimide sites in Proteins by N-terminal sequence analysis after alkaline hydroxylamine cleavage. *Protein Sci* 1994; 3: 147–9.
218. Beal JL, Foster SB, Ashby MT. Hypochlorous acid reacts with the N-terminal methionines of proteins to give dehydromethionine, a potential biomarker for neutrophil-induced oxidative stress. *Biochemistry* 2009; 48: 11142–8.
219. Miller BL, Kuczera K, and Schöneich C. One-electron photooxidation of N-methionyl peptides. Mechanism of sulfoxide and azasulfonium diastereomer formation through reaction of sulfide radical cation complexes with oxygen or superoxide. *J Am Chem Soc* 1998; 120: 3345–56.
220. Hillaert S, Van den Bossche W. Determination of captopril and its degradation products by capillary electrophoresis. *J Pharm Biomed Anal* 1999; 21: 65–73.
221. Moorhouse KG, Nashabeh W, Deveney J et al. Validation of an HPLC method for the analysis of the charge heterogeneity of the recombinant monoclonal antibody IDEC-C2B8 after papain digestion. *J Pharm Biomed Anal* 1997; 16: 593–603.
222. Battersby JE, Hancock WS, Canova-Davis E, Oeswein J, O'Connor B. Diketopiperazine: formation and N-terminal degradation in recombinant human growth hormone. *Int J Peptide Protein Res* 1994; 44: 215–22.
223. Niedernhofer LJ, Riley M, Schnetz-Boutaud N et al. Temperature-dependent formation of a conjugate between tris-(hydroxymethyl)aminomethane buffer and the malondialdehyde-DNA adduct pyrimidopurinone. *Chem Res Toxicol* 1997; 10: 556–61.
224. Bubb WA, Berthon HA, Kuchel P. Tris buffer reactivity with low molecular weight aldehydes, NMR characterization of the reactions of glyceraldehyde 3-phosphate. *Bioorg Chem* 1995; 23: 119–30.
225. Richter S, Fabris D, Binaschi M et al. Effects of common buffer systems on drug activity: the case of clerocidin. *Chem Res Toxicol* 2004; 17: 492–501.
226. Richter SN, Fabris D, Moro S, Palumbo M. Dissecting reactivity of clerocidin toward common buffer systems by means of selected drug analogues. *Chem Res Toxicol* 2005; 18: 35–40.
227. Harris CM, Kopecka H, Harris TM. Vancomycin: structure and transformation to CDP-I. *J. Am. Chem. Soc.* 1983; 105: 6915–22.
228. Zhou CC, Stoner EJ, Kristensen EW et al. Formation, isolation and characterization of an AB-biaryl atropisomer of oritavancin. *Tetrahedron* 2004; 60: 10611–18.
229. Clayden J, Moran WJ, Edwards PJ, LaPlante SR. The challenge of atropisomerism in drug discovery. *Angewandte Chemie, International Edition*. 2009; 48: 6398–401.
230. Clarke HT, Gillespie HB, Weisshaus SZ. The action of formaldehyde on amines and amino acids. *J Am Chem Soc* 1933; 55(11): 4571–87.

4 | Stress testing: Analytical considerations

Patrick J. Jansen, W. Kimmer Smith, and Steven W. Baertschi

STRESS-TESTING CONDITIONS AND SAMPLE PREPARATION

Introduction

Although there are some guidelines for stress testing given in the International Conference on Harmonization (ICH) guideline on the stability testing of drug substances and drug products, the guidance given is very general and not particularly useful for designing a stress-testing study. The following is an excerpt from the revised guideline [Q1A(R2)] (1).

Stress testing is likely to be carried out on a single batch of the drug substance. It should include the effect of temperatures [in 10°C increments (e.g., 50°C, 60°C, etc.) above that for accelerated testing], humidity (e.g., 75% RH or greater) where appropriate, oxidation, and photolysis on the drug substance. The testing should also evaluate the susceptibility of the drug substance to hydrolysis across a wide range of pH values when in solution or suspension. Photostability testing should be an integral part of stress testing.

Additional guidance is given only for photostability testing (2). Since there is very little guidance on stress testing, the goal of this chapter is to provide the reader with general guidance on the design, set up, and analytical aspects of carrying out stress-testing studies. The main focus of this chapter is chemical degradation; therefore, the assessment of the physical stability of solid drug substances is not discussed. Although a significant amount of detail is given in this chapter, the reader is reminded that these are only suggestions from the experience of the authors and that there are many acceptable ways to perform these studies.

The timing of the stress test screen will vary usually with drug substance testing in the early preclinical phase of development and drug product stress testing occurring later. See chapter 5 for more discussion on this topic. The early phase stress test screen results for drug substance can be quite valuable for formulators of the drug product as well as to those responsible for developing the analytical methods for the drug product.

Data Gathering

The first task before beginning stress-testing studies is to gather all the relevant information about the compound. Information such as molecular structure, solubility, $pK(s)$, known chemical instability, hygroscopicity, enantiomeric purity, etc., is important. In addition, previously established analytical methods may provide a starting point for development of more discriminating methods required for the separation of the complex mixtures which may result from stress testing.

The molecular structure of a compound is very important. For example, one can usually deduce from the structure whether or not the compound will absorb UV radiation and be detectable with a UV detector. The molecular structure also reveals if the compound has ionizable functional groups and will require a mobile phase modifier if HPLC analysis is used. Examination of the molecular structure may also tell something about the chemical reactivity of the molecule. The molecular structure indicates whether the molecule contains any chiral centers. If the molecule is chiral and nonracemic, then an assay to determine chiral stability may be required. Knowledge of the solubility of the compound, particularly the aqueous solubility, is required in order to design the study. If the aqueous solubility is too low, then a relatively inert organic cosolvent such as acetonitrile may be utilized to achieve solutions for stressing. Refer to Table 4 of chapter 2 for a listing of other potential cosolvents along with the pros and cons of using each solvent.

Table 1 Typical Stress Conditions for Preliminary Studies

Storage Condition	Time/Exposure
Aqueous solution/simulated sunlight	2–3 × ICH confirmatory exposure
0.1 N HCl solution/up to 70°C	1–5 days
0.1 N NaOH solution/up to 70°C	1–5 days
0.3% H ₂ O ₂ solution/ambient temperature in the dark	1–5 days
75/20/5 acetonitrile/H ₂ O/MeOH (The addition of methanol to the azo-initiator solutions is proposed to reduce the levels of undesired alkoxy radicals formed, see Ref. 3.) solution with radical initiator Vazo 52 30°C or AIBN 40°C	1–3 days

Designing Stress-Testing Studies

Preliminary Studies

Unless a significant amount of information about the stability of the molecule is known, it will probably be necessary to conduct some preliminary studies to gain some basic information about the stability of the compound. It is important to investigate the solubility of the material to be tested and to consider using a cosolvent such as acetonitrile (ACN) if the sample is not fully soluble in aqueous media. It is also important not to use sample solvent of considerably greater strength than the chromatographic conditions at which time the parent peak will elute. This can disrupt the chromatographic process resulting in poor peak shape.

Generally, the goal of stress testing is to facilitate an approximate 5–20% degradation of the sample under any given condition (if possible after reasonable limits of stressing).

In the preliminary investigation, observations are made regarding sample stability via exposure of solution samples to pH extremes, oxidative conditions including hydrogen peroxide and a radical initiator such as 2,2-azobisisobutyronitrile (AIBN, Vazo 64) or 2,2-azobis-(2,3-dimethylvaleronitrile) (Vazo 52). Light and heat may also be employed.

Table 1 lists some typical stress conditions for preliminary studies.

Stress-Test Screen

After conducting some preliminary studies and developing analytical methods (the development of appropriate stress testing methods is dealt within section “Methods of Analysis” of this chapter) it is time to design the stress test screen. Unfortunately, it is impossible to devise a universal set of stress conditions since there is significant variability in the stability of drug compounds. What can be defined, however, are suggested upper limits for the various stress conditions that can be used as starting points for stress-testing studies. If no degradation can be induced at these proposed maximum stress conditions, then it is concluded that the molecule is stable. Refer to chapter 2 for a discussion of the rationale used to establish the maximum stress conditions. While this chapter deals with drug substance, many of the same practices can be applied for drug product testing. See chapter 2 for further discussion of drug product stress testing considerations. Although this chapter focuses on manual preparation of samples and their analysis, automated systems have been developed to facilitate the entire stress testing process including sample dilution, storage in appropriate environmental conditions, and sampling for assay (see chap. 21). While considerable expense is involved in an automated system, it can greatly increase throughput over the manual approach if required.

DESIGN OF STUDY

Taking into account the information derived from the results of the preliminary study, one can devise a more detailed stress test study. See Tables 3, 6, and 7–10 in chapter 2 for proposed conditions and analytical time points for stress testing. Additional conditions can be added if deemed necessary.

Sample Preparation General

Solid-State Samples

Solid-state samples can be prepared by accurately weighing the drug substance into a container that can be stored under the appropriate condition. Suitable containers include volumetric flasks, scintillation vials, etc. The amount of drug substance used is usually dictated by the availability of material, accuracy of balances, and the final concentration desired for analysis. Typical amounts used for solid-state samples would be between 2 and 20 mg. Weighing of samples may also be achieved by the use of an automated process such as using a Powder-nium™ workstation. See chapter 21 for further discussion of automation. For example, if the final analytical concentration desired is 0.3 mg/mL, solid-state samples of ~3 mg in 10-mL volumetric flasks could be stressed and then simply diluted to volume at the time of assay. Alternatively, samples could be prepared in scintillation vials, stressed, and then diluted with a known amount of solvent. Solid-state samples can be preweighed into the respective containers before stressing. Prew weighing the samples simplifies the analysis of stressed samples by eliminating any concerns about changing levels of volatile constituents such as water or organic solvents during thermal stress. The ICH guideline on photostability indicates that samples for photoexposure be less than 3 mm in depth (2).

Occasionally, one will have to deal with hygroscopic drug substances or drug substances containing a significant level of a volatile compound (e.g., solvates). These types of drug substances can pose significant issues when preparing samples for quantitative analysis. Fortunately, most of these issues can be overcome by using a simple approach. A simple method for eliminating volatile content issues is to allow samples to come to equilibrium with the environment and then conducting all of the weighing of samples and standards over as short a time frame as possible. Performing a volatiles analysis (i.e., TGA) both before and after sample weighing will provide assurance that no significant change in volatiles content occurred during the weighing period.

Solution Samples

Solution samples can be prepared in a number of different ways. One way is to prepare a single stock solution at a known concentration for each stress condition and then pull aliquots for analysis at the desired time points. This method requires that the container used for the solution be tightly closed to prevent evaporation. If evaporation is a problem, it can be overcome by preparing a separate sample for each time point at a concentration higher than the analytical concentration. For example, if the final analytical concentration desired is 0.3 mg/mL, solutions could be prepared at a concentration of ~1 mg/mL by adding 3 mL of the appropriate solvent to samples of ~3 mg in 10-mL volumetric flasks. Prior to assay, the samples can then be diluted to volume to achieve the final analytical concentration.

Suspension or Slurry Samples

Suspensions or slurries pose a problem since by definition they are not homogeneous. The problem is how to obtain reliable quantitative results from suspensions. One method for dealing with suspensions is to prepare individually weighed samples and stress them at concentrations greater than the final analytical concentration. Prior to analysis the samples are then diluted to the final analytical concentration with a solvent that completely dissolves the sample. For example, if the final analytical concentration desired is 0.3 mg/mL, suspensions could be prepared at a concentration of ~1 mg/mL by adding 3 mL of the appropriate solvent to samples of ~3 mg in 10-mL volumetric flasks. Prior to assay, the samples can then be diluted to volume with a solvent capable of completely dissolving the sample.

Standards

The assay of stressed samples will usually require the use of some type of external standard. The external standard could be an established reference standard; however, the preferred

method is to use the same material/lot as is being stressed. This is easily accomplished by weighing additional samples (that will not be stressed) for use as “standards” at the same time as the stress test samples are weighed. The “standards” should then be stored under conditions that will assure that no degradation will occur (e.g., freezer). At the time of analysis, the stressed samples are simply assayed versus the freshly prepared unstressed “standards” and the results calculated as percent initial.

Solution and Buffer Preparation

Typically, 0.1 N HCl and 0.1 N NaOH are used for the pH extremes of aqueous solution stressing (i.e., pH 1 and 13, see chap. 2). Since neither of these solutions possesses significant buffering capacity, the pH of the solution should be verified following addition of the drug to these solutions. In order to obtain solutions at pH values between 1 and 13, a buffer must be used. It is desirable to use the same buffer for all the pH levels to avoid chemical differences between different buffers, since buffers are not always inert and can sometimes act as catalysts for drug degradation or even react with the drug being studied(4). Unfortunately, no single buffer provides buffering capacity across this wide pH range. A common practice is to make the buffer of sufficient ionic strength such that it still offers some pH stability even outside of its normal buffering range (e.g., 50 mM phosphate). For example, if pH values of 3, 5, 7, 9, and 11 are desired, a phosphate buffer can be used keeping in mind that the buffering capacity will be low at pH 5 and pH 9. If more buffering capacity is required, then other buffers or a combination of buffers can be used. The buffering range for several common buffers is given in Table 2.

Example Stress Test Screen

The following sections describe a stress-testing study conducted on LY334370 hydrochloride. The structure of LY334370 hydrochloride is shown in Figure 1. This stress-testing example illustrates many of the concepts discussed in the previous paragraphs. An important detail that should be pointed out is that this work did not include the use of transition metals [e.g., iron(III) or copper(II)]; therefore, no results from these conditions are given.

Data Gathering

Examination of the structure of the example compound clearly indicates that it possesses both a phenyl and an indole moiety and should therefore be amenable to UV detection. The compound has a tertiary amine, which is an ionizable functional group with a pK_a of ~ 9 , therefore,

Table 2 Some Common Buffers and Their Buffering Ranges

Buffer	pK_a	Buffer Range
Phosphate	2.1	1.1–3.1
	7.2	6.2–8.2
	12.3	11.3–13.3
Citrate	3.1	2.1–4.1
	5.4	4.4–6.4
Formate	3.8	2.8–4.8
Succinate	4.2	3.2–5.2
	5.6	4.6–6.6
Acetate	4.8	3.8–5.8
Citrate	3.1	2.1–4.1
	4.7	3.7–5.7
	5.4	4.4–6.4
Tris	8.3	7.3–9.3
Borate	9.2	8.2–10.2

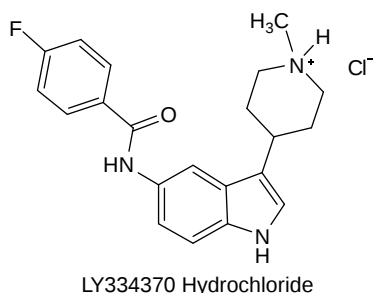


Figure 1 The chemical structure of LY334370.

Table 3 Semiquantitative Solubility Study Result

Solvent	Solubility (mg/mL)
Water	>2.75
Methanol	>5
Acetonitrile	<0.5
Water/acetonitrile 50/50 (v/v)	>4.4
Water/acetonitrile 80/20 (v/v)	>4.4
0.1 N HCl	<0.5
pH 2 phosphate buffer	>3.2
pH 4 phosphate buffer	>2
pH 6 phosphate buffer	>1.5
pH 8 phosphate buffer	<0.5
0.1 N NaOH	<0.5

a buffered mobile phase will likely be required if HPLC is chosen as the analytical method. Since the compound does not contain a chiral center, there is no need for chiral analysis. Since there was no available solubility information on LY334370 hydrochloride, a simple and semiquantitative solubility study was performed.

Table 3 gives the results of this study. The solubility values were obtained by adding a specified amount of solvent to a known quantity of the drug and visually observing whether all the material dissolved. Although the solubility numbers are only rough estimates of solubility, they are very useful for designing the stress-testing study. The solubility results indicate that an organic cosolvent will be necessary to achieve solutions at pH values of 8 and higher and in 0.1 N HCl.

Preliminary Studies

In the case of LY334370 hydrochloride, an analytical method needed to be developed and very little was known about its degradation chemistry. Solid-state and solution samples of LY334370 hydrochloride were prepared and stressed under the conditions described in Table 4. The samples were then analyzed by HPLC using the preliminary HPLC conditions given in Table 9. Calculation of the area percent of the impurity peaks provided an estimate of the amount of degradation that occurred in each sample. The results indicate that LY334370 appears to be stable in the solid state but degrades significantly under acidic, basic, and oxidative conditions. Some of the degraded samples generated in this preliminary study were used to further develop the analytical method used for the final study. More details on the development of the method can be found in the next section. The results of this preliminary study were also used to design the time points and conditions of the final stress testing study where the objective was to induce 5–20% degradation, if possible.

Table 4 Preliminary Sample Descriptions and Results

Sample Description	Stress Condition	Result-Related Substances Increase (%)
Approx. 2 mg in an open 10-mL volumetric flask	70°C/uncontrolled RH for 21 days	0.0
Approximately 2 mg in an open 10-mL volumetric flask, which was stored in a sealed container over a saturated NaCl solution	70°C for 21 days (75% RH)	0.0
Approximately 2 mg in a 20-mL scintillation vial covered with the polyethylene film	Irradiate with simulated sunlight produced by a xenon arc lamp for 20 hr. Visible exposure: ~3 million lux-h UV Exposure: ~1500W h/m ²	0.0
Approximately 2 mg dissolved in 2 mL of 70/30 0.1 N HCl/acetonitrile	70°C for 7 days	20.4
Approximately 2 mg dissolved in 2 mL of water	70°C for 7 days	0.2
Approximately 2 mg dissolved in 2 mL of 70/30 pH 8 phosphate/acetonitrile	70°C for 7 days	10.7
Approximately 2 mg dissolved in 2 mL of 70/30 0.1 N NaOH/acetonitrile	70°C for 7 days	27.0
Approximately 2 mg dissolved in 2 mL of 0.3% hydrogen peroxide	Ambient temperature protected from light for 7 days.	2.2
Approximately 2 mg of drug and a molar equivalent of AIBN dissolved in 2 mL 20/80 water/acetonitrile	40°C for 7 days	16.5
Approximately 2 mg dissolved in 2 mL of water	Irradiate with simulated sunlight produced by a xenon arc lamp for 20 hr. Visible Exposure: ~3 million lux-h UV Exposure: ~1500W hr/m ²	8.1

Stress-Testing Study

The results of the preliminary study were used to design the final stress testing study. Examination of the preliminary solid-state results indicates that the molecule appears to be stable under all of the solid-state conditions and therefore can be stressed at the maximum temperature (70°C) with a minimum number of time points. The preliminary solution results indicate that the molecule is susceptible to degradation particularly at the pH extremes at the upper temperature limit of 70°C. The temperature of 70°C appears to be appropriate for most conditions but will require time points shorter than 7 days. The final screen was designed keeping these preliminary results in mind. Tables 6–9 list the final stress-testing conditions, time points, and results. The HPLC method used for the final screen is given in Table 5.

Experimental Details

Solid-state samples were prepared in duplicate and consisted of ~3 mg of material accurately weighed into a 10-mL volumetric flask. Samples were subjected to heat at 70°C under both ambient and high humidity. High humidity conditions were maintained by storing samples in open flasks over a saturated NaCl/H₂O solution in a closed glass container. Solid-state samples were exposed to simulated sunlight generated using a xenon arc lamp in a separate experiment. These samples consisted of ~3 mg of material in a clear 20-mL scintillation vial that was sealed with Glad Wrap™ (i.e., polyethylene film). In all cases, the thickness of the samples was significantly less than 1 mm. Control samples were prepared in the same manner except that they were completely wrapped in aluminum foil.

Table 5 HPLC Screening Method Used to Analyze Preliminary Samples

Buffer	0.025 M KH_2PO_4 , adjust pH to 2.5 with H_3PO_4	
Mobile phase A	97/3 buffer/ACN	
Mobile phase B:	25/75 buffer/ACN	
Column	250 × 4.6 mm Zorbax SB-C18 5.0 μm , 40°C	
Detection	UV-PDA	
Sample solvent	77/23 buffer/ACN	
Sample concentration	~0.3 mg/mL in sample solvent	
Injection volume	10 μL	
Flow	1.5 mL/min	
Gradient	Time	%B
	0.0	0
	5	15
	20	25
	30	100

Abbreviation: ACN, Acetonitrile.

Table 6 LY334370 Hydrochloride Solid State Stress Conditions and Results

Storage Condition	Container	Time Point	Assay (% Initial)	Related Substances Increase (%)	Physical Appearance
70°C	Open 10-mL volumetric flask	14	98.0	0.1	No change
		28	98.4	0.1	No change
70°C over saturated NaCl	Open 10-mL volumetric flask	14	99.1	0.1	No change
		28	99.4	0.1	No change
Simulated sunlight	Glad Wrap sealed 20-mL scintillation vial	20 hr	99.3	0.3	No change
Simulated sunlight (control)	Glad Wrap sealed 20-mL scintillation vial wrapped with foil	20 hr	99.7	0.3	No change

The solution and slurry samples were prepared in duplicate and consisted of ~3 mg of material accurately weighed into a 10-mL volumetric flask. Three milliliters of the appropriate solvent was added to each sample and the flasks were tightly stoppered prior to placing the flask in the given storage condition. Immediately prior to analysis, after equilibration to room temperature, all samples were diluted to volume with 77/23 (v/v) 0.025 M KH_2PO_4 pH 2.0 buffer/ACN. The solution samples could have been prepared as stock solutions with removal of a sample aliquot at the appropriate time points; however, evaporation of the solution at the stress temperature (70°C) was a major concern. Preparation of individual slurry/suspension samples that were fully dissolved just prior to analysis eliminated the evaporation concern.

All the buffers (pH 2, 4, 6, 8) were prepared from 0.05 M KH_2PO_4 adjusted to the proper pH with either 5 N NaOH or 85% H_3PO_4 . The 0.3% H_2O_2 solution was prepared by diluting 10 mL of fresh 3% H_2O_2 to 100 mL with water. The AIBN solutions contained ~1 molar equivalent of AIBN with respect to LY334370 hydrochloride.

The photostability chamber was a Suntest CPS+ manufactured by Atlas Material Testing Technology, LLC, Chicago, IL. It was set up to simulate natural sunlight and contained a xenon long-arc lamp with an infrared filter and a UV filter with a cutoff of ~295 nm. The photostability chamber was set at an intensity of 765 W/m² (300–800 nm). Manufacturer measurements

Table 7 LY334370 Hydrochloride Solution and Slurry Stress Conditions and Results

Storage Condition	Container	Time Point (Days)	Assay (% Initial)	Related Substances Increase (%)	Physical Appearance
Slurry in 0.1 N HCl at 70°C	Closed 10-mL volumetric flask	3	92.6	5.8	Faint pink, clear
		7	81.5	14.2	Faint pink, clear
		14	60.6	27.4	Amber, clear
Solution in 80/20 0.1 N HCl/ACN at 70°C	Closed 10-mL volumetric flask	3	96.7	2.0	Faint pink, clear
		7	90.8	5.9	Faint pink, clear
		14	71.3	14.8	Amber, clear
Solution in pH 2 phosphate buffer at 70°C	Closed 10-mL volumetric flask	3	99.4	0.5	Faint pink, clear
		7	98.4	1.0	Faint pink, clear
		14	93.8	3.1	Faint yellow, clear
Solution in pH 4 phosphate buffer at 70°C	Closed 10-mL volumetric flask	3	100.4	0.1	No change
		7	99.8	0.1	No change
		14	98.9	0.2	No change
Solution in unbuffered water at 70°C	Closed 10-mL volumetric flask	3	99.8	0.2	No change
		7	100.0	0.1	No change
		14	97.1	1.0	Faint yellow, clear
Solution in pH 6 phosphate buffer at 70°C.	Closed 10-mL volumetric flask	3	99.9	0.0	Faint yellow, clear
		7	100.1	0.2	Faint yellow, clear
		14	99.0	0.5	Faint yellow, clear
Slurry in pH 8 phosphate buffer at 70°C	Closed 10-mL volumetric flask	3	99.2	0.6	Faint yellow, clear
		7	95.2	1.7	Faint yellow, clear
		14	87.7	8.2	Yellow, clear
Solution in 80/20 pH 8 phosphate buffer/ACN at 70°C	Closed 10-mL volumetric flask	3	95.5	3.0	Faint pink
		7	89.3	6.3	Faint yellow, cloudy
		14	81.9	10.6	Yellow, clear
Slurry in 0.1 N NaOH at 70°C	Closed 10-mL volumetric flask	3	63.9	21.1	Amber, cloudy
		7	40.5	20.2	Amber, cloudy
		14	6.8	32.7	Dark amber, ppt
Solution in 80/20 0.1 N NaOH/ACN at 70°C	Closed 10-mL volumetric flask	3	91.1	5.2	Faint yellow, clear
		7	88.8	9.0	Faint yellow, cloudy
		14	85.6	13.4	Faint yellow, cloudy

Abbreviation: ACN, Acetonitrile.

indicate that this setting corresponds to a visible intensity of ~150,000 lx, and a UVA intensity (320–400 nm) of ~78 W/m². Thus, a 10 hour sample exposure corresponds to ~1.5 million lx hour visible and ~780 W hr/m² UVA. For comparison, the ICH guideline on photostability specifies a minimum exposure for confirmatory studies of 1.2 million lx hour in the visible and not less than 200 W hr/m² in the near UV.

Table 8 LY334370 Hydrochloride Solutions Oxidative Conditions and Light Exposure

Storage Condition	Container	Time Point	Initial (%)	Related Substances Increase ($t-t_0$)	Physical Appearance
Solution in 0.3% H ₂ O ₂ stored in dark	Closed 10-mL volumetric flask stored in the dark	3	98.9	0.8	No change
		7	97.4	1.6	No change
		14	96.7	3.2	No change
Solution in 80/20 ACN/H ₂ O containing AIBN at 40°C	Closed 10-mL volumetric flask	3	87.9	8.8	Faint yellow, clear
		7	74.7	18.2	Faint yellow, clear
		14	58.9	25.6	yellow, clear
Solution in unbuffered water exposed to fluorescent light at an intensity of ~16,000 lux	Closed 10-mL volumetric flask	14	80.2	19.3	No change
		28	64.6	33.6	No change
Solution in unbuffered water exposed to fluorescent light at an intensity of ~16,000 lux (control)	Foil wrapped closed 10-mL volumetric flask	14	99.9	0.0	No change
		28	99.1	0.4	No change
Solution in pH 2 buffer irradiated with simulated sunlight	Closed 10-mL volumetric flask	10 hr	96.7	0.9	Faint tan, clear
Solution in pH 2 buffer irradiated with simulated sunlight (control)	Foil wrapped closed 10-mL volumetric flask	10 hr	99.3	0.3	No change
Solution in unbuffered water irradiated with simulated sunlight	Closed 10-mL volumetric flask	10 hr	95.5	6.7	No change
Solution in unbuffered water irradiated with simulated sunlight (control)	Foil wrapped closed 10-mL volumetric flask	10 hr	100.2	0.3	No change
Solution in pH 6 buffer irradiated with simulated sunlight	Closed 10-mL volumetric flask	10 hr	97.4	2.6	No change
Solution in pH 6 buffer irradiated with simulated sunlight (control)	Foil wrapped closed 10-mL volumetric flask	10 hr	100.5	0.2	No change

Table 9 Final HPLC Conditions Developed for Stress Testing of LY334370

Mobile Phase A	0.025 M KH ₂ PO ₄ Adjust pH to 2.0 with H ₃ PO ₄	
Mobile phase B	acetonitrile	
Column:	250 × 4.6 mm Zorbax SB-Ph 5.0 mm, 40°C	
Detection	UV-PDA at 205 nm (260 nm for assay)	
Flow	1.0 mL/min	
Gradient	Time (min)	Acetonitrile (%)
	0.0	3
	5	15
	25	27
	35	75

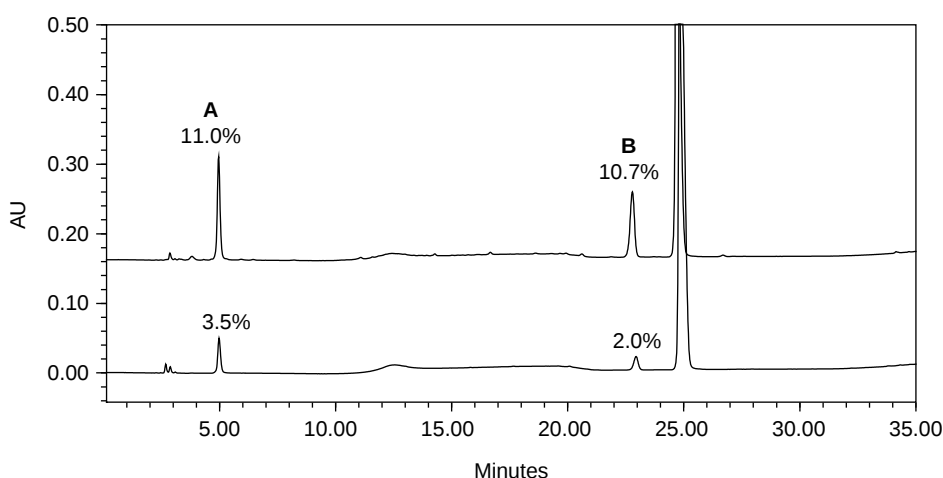


Figure 2 HPLC related substances chromatograms (UV detection, 205 nm) obtained on slurries of LY334370 hydrochloride in 0.1 N NaOH (*top*) and 0.1 N HCl (*bottom*) held at 70°C for 3 days.

The fluorescent light chamber consisted of 8 Sylvania fluorescent bulbs (Octron, 4100 K, 32 W) in a 19 in × 21 in × 50 in box. The average intensity of the light with measurements taken at the level of the samples was ~16,000 lx. The average temperature of the light box was ~26°C. Cool white fluorescent light exposure was included in the study in addition to simulated sunlight to enable the assessment of the potential for photodegradation from visible light exposure only (e.g., in the laboratory environment, manufacturing facilities, etc.). The stressed samples were assayed versus unstressed LY334370 hydrochloride prepared at concentrations corresponding to ~50%, 80%, and 110% of the nominal concentration of the samples (0.3 mg/mL). A three-point standard curve was constructed using the standards and the concentration of LY334370 in the stressed samples was determined using the curve. All samples and standards were weighed out at the same time to eliminate any concerns about volatiles content changes during stressing. The undiluted standards were stored in a refrigerator at ~5°C prior to use. TGA analyses run on this material prior to the weighing of samples and following the weighing of samples indicated no change in volatile content during the weighing process. The related substances results were calculated versus an external standard prepared at a concentration of ~1% (0.003 mg/mL) of the nominal sample concentration using the equation given below. Total related substances on the unstressed material were measured to be 0.34%. All of the related substances results were corrected for this initial related substances level and are reported as related substances increase.

$$\% \text{ Rel Subs} = \frac{(\text{Area of Related Substances}) \times (\text{Concentration of Standard}) \times 100\%}{(\text{Area of Standard}) \times (\text{Concentration of Sample})}$$

Results

LY334370 hydrochloride was stable under all of the solid-state conditions studied. No significant loss of potency or increase in related substances was detected for any of the solid-state samples. These results suggest that LY334370 hydrochloride should be stable under typical ambient conditions and should not require any special handling or storage conditions. Since no significant degradation occurred in the solid-state samples, none of the solid-state chromatograms are shown.

Selected chromatograms of suspension and solution samples are shown in Figures 2–5. Examination of Figure 2 indicates that LY334370 undergoes degradation to two major degradation products (A and B) at the pH extremes of 0.1 N HCl and 0.1 N NaOH.

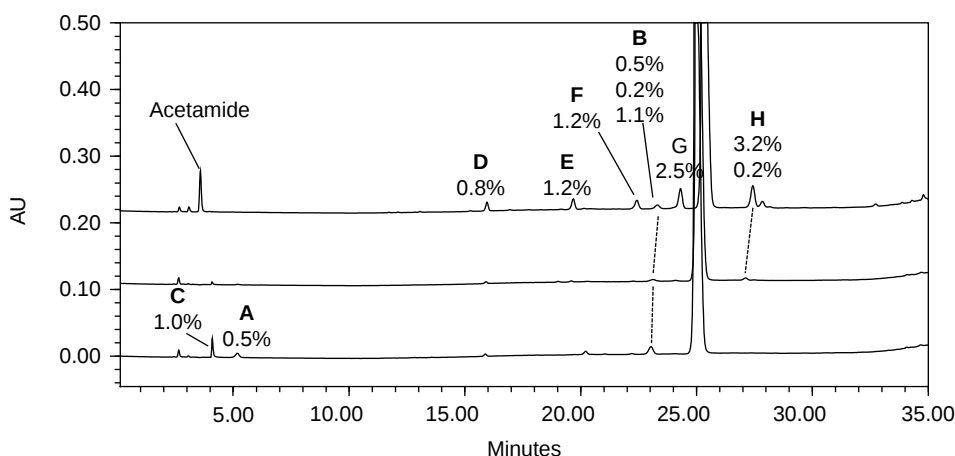


Figure 3 HPLC related substances chromatograms (UV detection, 205 nm) obtained on solutions of LY334370 hydrochloride in pH 2 phosphate buffer (bottom), water (middle), and pH 8 phosphate buffer/acetonitrile (top) held at 70°C for 14 days.

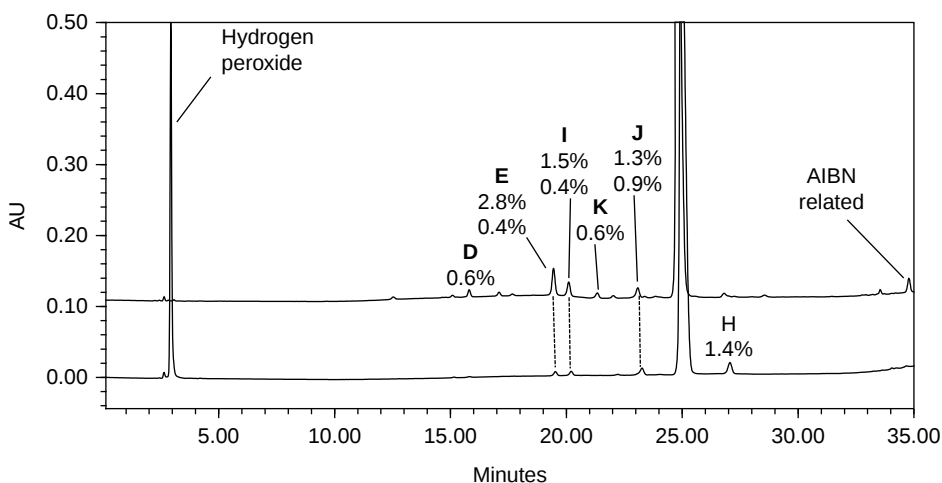


Figure 4 HPLC-related substances chromatograms (UV detection, 205 nm) obtained on solutions of LY334370 hydrochloride in 0.3% hydrogen peroxide at ambient temperature for 14 days (bottom) and a water/acetonitrile solution containing the radical initiator AIBN held at 40°C for three days.

It is likely that peaks A and B are the result of hydrolysis, but confirmation of this assumption would require characterization of these degradation products. The chromatograms in Figure 3 indicate that LY334370 is relatively stable at pH 2 and in unbuffered water but does degrade to a number of new peaks in pH 8 buffer containing a cosolvent. Some degradation is apparent in solutions containing hydrogen peroxide or a radical initiator (Fig. 4), suggesting that LY334370 may be susceptible to oxidative degradation.

The chromatograms shown in Figure 5 suggest that solutions of LY334370 are susceptible to light-catalyzed degradation. The stress testing studies on LY334370 indicate that the major potential degradation products are peaks A through K. Which of these peaks are relevant degradation products (i.e., form under normal storage conditions) is determined using the results of formal stability studies.

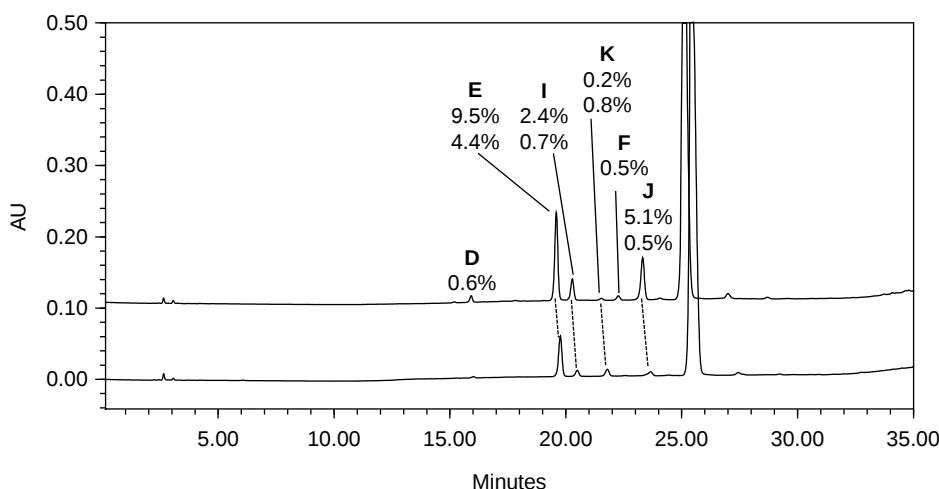


Figure 5 HPLC related substances chromatograms (UV detection, 205 nm) obtained on solutions of LY334370 hydrochloride in water exposed to intense fluorescent light for 14 days (*bottom*) or simulated sunlight produced by a xenon arc lamp (*top*).

METHODS OF ANALYSIS

Introduction

When developing a method for stress-testing studies, it is useful to think about what would constitute an ideal method. An ideal method would enable the accurate quantification of the parent compound as well as all of its degradation products. While this sounds simple in theory, it is nearly impossible to develop an ideal method early in the development cycle of a new drug when few if any of the potential degradation products are known. After all, one of the reasons for conducting stress-testing studies is to discover the potential degradation products. The ideal chromatographic method will resolve all degradation products from the parent as well as from each other, all degradation products will be detected, and the relative response of the degradation products with respect to the parent will be known. Since it is difficult to develop a chromatographic method that satisfies all of these requirements, the focus should be on developing a primary screening method that has the highest likelihood of resolving and detecting a diverse set of degradation products. In many cases, it may be impossible to come up with an ideal method; however, the use of two “orthogonal” analysis methods often will give adequate results in these cases.

Specific versus Generic Methods

Chromatographic methods used for analysis of stress-testing samples can be developed for each specific drug substance or product being stressed or they can be “generic” methods, which are used for analysis of samples from many drug substances. The major advantage to using a generic method is the reduced amount of method development time for each compound. This is something that can be particularly important for laboratories that conduct stress testing on many compounds such as early-phase development labs. Some of the disadvantages include a less-specific method and the greater likelihood that major degradation products may be missed. If one is developing a generic HPLC method for stress testing, gradient elution will almost certainly be required since different drug substances likely will have different polarities. In our experience, we have found that just a few reversed-phase methods work for greater than 80% of the compounds for which we have conducted stress-testing studies. These generic method conditions are outlined in Table 10. Refer to the reversed-phase HPLC discussion later in this chapter for more information on selection of chromatographic parameters.

Table 10 Generic Reversed-Phase HPLC Conditions

Column	C ₈ or C ₁₈
Weak solvent	0.1% trifluoroacetic acid in water Phosphate buffer, pH 2–3 for acids or pH 6–7 for bases Ammonium acetate, pH 4.5 to 5.5 (works well when using MS detection)
Strong solvent	Acetonitrile or methanol
Column	C ₈ or C ₁₈
Detection	UV-PDA
Elution	Linear gradient adjusted such that the parent elutes near the middle of the chromatographic run

Validation of Methods

According to the ICH guideline on validation of analytical methods (5), the objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. The reader should keep in mind that stress-testing methods are screening methods to be used to help understand the degradation chemistry of a drug and therefore do not need to (nor, in general, can they) be validated to the extent of final control methods. In addition, stress-testing methods are usually only used in a limited number of laboratories without a formal method transfer. The overall validation should be significantly abbreviated when compared to the validation of final control methods, since stress-testing methods are investigational methods. The concepts in the ICH guideline on validation of analytical methods are a good starting point for validation of stress testing methods. The ICH guideline gives parameters to be considered when validating methods. These parameters include accuracy, precision, specificity, detection limit, quantitation limit, linearity, and range. All of these parameters should be addressed to some extent when validating stress-testing methods; however, the overall validation should be kept to a minimum since stress-testing methods are investigational methods.

Accuracy normally should not be a problem with stress testing methods as long as the method is linear and samples are completely dissolved prior to analysis. The specificity of methods cannot be fully validated since one normally does not know all of the possible degradation products during initial stress testing. Specificity can be addressed by using any known impurities and the degradation products produced in the method development samples. Precision (repeatability) of the assay of the main component can be evaluated by preparing a limited number of assay samples (e.g., 5 to 10) and using simple statistics to estimate the standard deviation. Estimation of intermediate precision and reproducibility should normally not be necessary for stress testing methods. Detection and quantitation limits for degradation products can be determined by using the parent compound and assuming that the responses of degradation products will be similar. Although there is no requirement to reach any specific detection limit a reasonable goal is 0.1%, since the goal of stress testing is to detect the major degradation products in samples ~10–20% degraded. The linearity of the method should be validated over ranges for both assay and impurity determination. A typical assay range might be from 50% to 110 % of nominal sample concentration while a typical range for impurity determination might cover a range from the quantitation limit to a few percent. If one wishes to quantitate impurities versus the parent peak then linearity (range) should be demonstrated from the quantitation limit to at least 100% of nominal sample concentration.

Chiral Molecules

Chiral analysis is required for drugs being developed as single enantiomers possessing a single chiral center capable of undergoing racemization. Molecules that have multiple chiral centers may not necessarily require a chiral method since racemization of one of the chiral centers will result in the formation of a diastereomer. Diastereomers can typically be resolved on achiral chromatography systems, while chiral impurities from a molecule with a single chiral center

will require a dedicated chiral method for analysis (6). Aside from chiral HPLC methods, chiral capillary electrophoresis (CE) is a commonly used technique (7).

Chromatographic Methods

There are numerous analytical techniques that can be used to analyze stress test samples. Some of the more common ones include reversed-phase HPLC, normal-phase HPLC, thin layer chromatography (TLC), CE, and gas chromatography (GC). The following paragraphs contain brief discussions on the use of these techniques for analysis of stress test samples.

Reversed-Phase HPLC

Since a large majority of pharmaceutical products are amenable to reversed-phase HPLC, this is usually the method of choice. The development of reversed-phase HPLC [or ultra high pressure liquid chromatography (UHPLC)] methods is a broad subject with many research articles and books devoted to it and it is not practical to try to cover this topic in depth in this chapter. There are, however, some major points to consider when developing an HPLC/UHPLC method for analyzing stress test samples (8).

HPLC versus UHPLC

UHPLC is becoming the standard in many modern analytical laboratories. UHPLC systems allow for rapid analyses (typically 5 minutes or less) on smaller columns with smaller (sub 2 μm) particle sizes. These systems can handle the higher pressures associated with smaller particles while also delivering higher numbers of theoretical plates (9). Although UHPLC requires different hardware, the development of UHPLC methods is really no different than the development of HPLC methods and the same factors must be considered.

Isocratic versus Gradient Elution

Since one does not know what degradation products will form, gradient elution should be used. This significantly increases the chances that degradation products, which are much more polar than the parent compound will be pulled away from the solvent front and those which are much less polar than the parent will elute from the column. Often the use of a multistep gradient is particularly beneficial. The first segment of the multistep gradient starts at very low organic concentration and ramps rapidly to the second segment in which the parent is eluted either under essentially isocratic conditions or with a very shallow gradient in order to maximize resolution of the degradation products from the parent compound. The third segment of the gradient begins after the parent elutes and is a rapid ramp to high organic concentration to elute any less polar degradation products. The following example illustrates the benefits of using gradient elution for stress testing. Figure 6 illustrates a chromatogram obtained under isocratic conditions on a sample of drug substance in 50/50 acetonitrile/pH 7 phosphate buffer stressed at 70°C for 14 days. The assay result of 95.1% indicates that significant degradation has occurred. The related substances result of only 0.7%, however, suggests a potential mass balance problem. Reanalysis of the same sample using gradient elution enabled the detection of a number of degradation products not visible with the isocratic method (Fig. 7).

Column Selection

For the majority of pharmaceutical products, C_8 or C_{18} bonded phases will give adequate separations and one of these two stationary phases should be tested before moving to other stationary phases. Sometimes improved selectivity of the separation can be achieved using other nonpolar stationary phases such as phenyl. Phenyl stationary phases are used since these phases can provide both hydrophobic interactions as well as π - π stacking interactions with double bonds and aromatic groups. Occasionally, polar analytes cannot be adequately retained on C_{18} or C_8 phases. In these cases the use of mixed-mode, polar-embedded stationary phases can be very useful. Polar embedded phases contain a polar group that is embedded

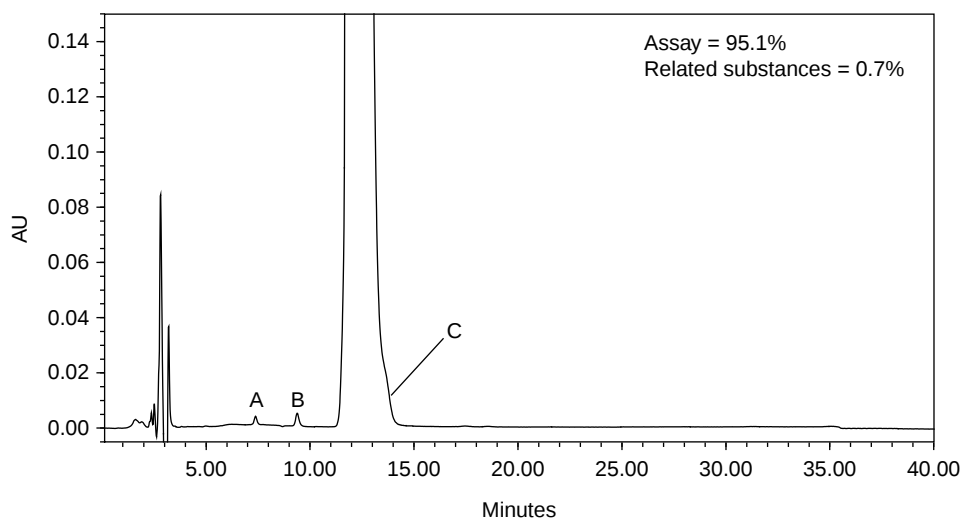


Figure 6 Isocratic HPLC chromatogram obtained on a sample of drug substance stressed in pH 7 phosphate buffer/ACN.

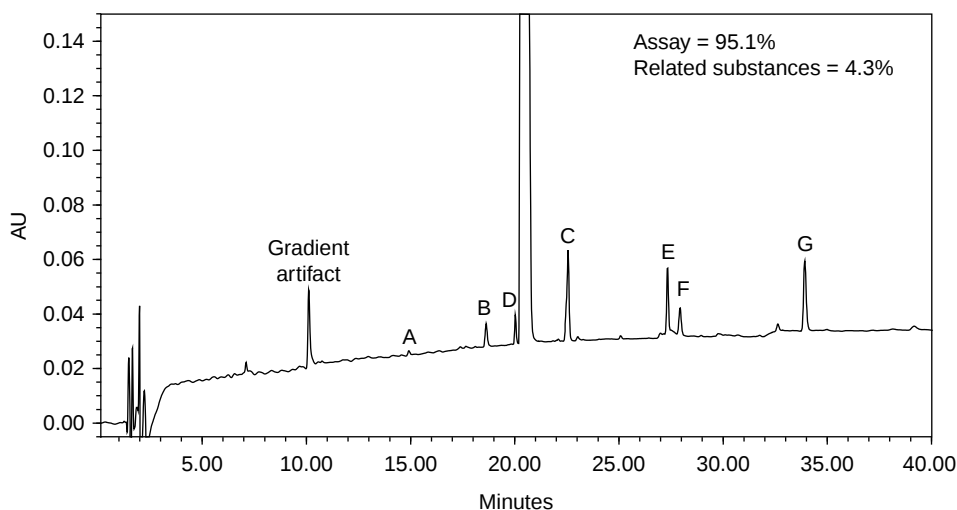


Figure 7 Gradient HPLC chromatogram obtained on a sample of drug substance stressed in pH 7 phosphate buffer/ACN.

within the nonpolar ligand chain. Common polar groups used in these phases include ethers, acrylates, carbamates, amides, and urea. The polar-embedded phases also can be useful for obtaining separation of compounds that cannot be separated using conventional C_8 or C_{18} stationary phases.

Mobile Phase Selection

There are a number of factors to consider when selecting a mobile phase. For compounds with ionizable functional groups, it is important that the pH of the mobile phase is controlled. The type of mobile phase additive used depends upon the type of detector used.

Detectors such as MS and CAD require the use of volatile mobile phase additives. Some of the more common volatile mobile phase additives include formic acid, trifluoroacetic acid, acetic acid, ammonium acetate salts, ammonium bicarbonate, and ammonium hydroxide. These mobile phase additives also work with UV or PDA detectors as well although some of them tend to obscure the lower wavelength range due to absorbance in the 200 to 220 nm range. When there is a desire to obtain chromatograms at lower wavelengths, phosphate buffers are a good choice due to their very low UV cutoffs. In addition, phosphate has three pK values and possesses good buffering capacity at low, neutral, and basic pH values. The organic component of the mobile phase is typically either acetonitrile or methanol. Acetonitrile offers some minor advantages over methanol like a lower UV cutoff and lower viscosity, but methanol typically works nearly as well.

Detection

There are many types of HPLC detectors available today, with the most popular ones including UV and UV-photodiode array (PDA), fluorescence, refractive index, evaporative light scattering (ELSD), charged aerosol (CAD), and the mass spectrometer. Of these, the most commonly used detector for pharmaceutical analytical methods is the UV detector since a majority of pharmaceutical compounds have some type of chromophore. Multiple detectors in series can also be utilized in order to obtain more information per chromatographic run. For example, a PDA detector can be combined with a mass detector to give both UV and mass spectral information on impurities (10). The use of a mass detector (LC-MS) contributes to the structure-elucidation process for degradation products in that structures may be proposed based on mass. For compounds that do not have any UV absorbance, the ELSD, mass spectrometric, and CAD detectors are generally the most useful and can be incorporated individually or in series.

The CAD is a detector that is described as a nearly universal detector for nonvolatile and semivolatile compounds, with a response that is reflective of the total mass passing through the detector and has been shown to be useful for the determination of response factors (11). Fluorescence detection is not desirable because there is no guarantee that the degradation products of a fluorescent molecule will fluoresce. Most refractive index detectors are best suited for isocratic elution and are therefore not particularly useful for stress-testing methods that utilize gradient elution. Since the UV detector is the most widely used, the remainder of this discussion will focus on the aspects of UV detection. The use of UV-transparent buffers and organic modifiers (e.g., phosphate buffers and acetonitrile or methanol) for the HPLC mobile phase is desirable since it enables chromatograms to be acquired at relatively low wavelengths near 200 nm. Typically, monitoring at these low wavelengths increases the likelihood that all of the degradation products will be detected since most compounds possessing a chromophore will absorb at these low wavelengths. The use of a PDA-UV detector significantly increases the amount of information obtained from the chromatographic run. The use of the PDA detector allows for the extraction of chromatograms at multiple wavelengths from a single chromatographic run as well as providing the UV spectra of individual peaks. These UV spectra can be used to correlate peaks arising from different stress conditions or from different chromatographic systems. The use of a PDA detector also enables the determination of the UV homogeneity of individual peaks, thereby giving an indication of their purity.

Normal-Phase HPLC

Normal-phase HPLC is a good complementary technique to reversed-phase HPLC in that it often gives different selectivity. It is also more effective in separating geometric isomers than reversed-phase HPLC. The main problem with normal-phase HPLC is that aqueous samples are not normally compatible with the technique. Since many of the stress-testing samples contain water, normal-phase HPLC is rarely used as the primary analytical technique for stress test samples. Nonetheless, normal phase can be a useful complementary technique to

reversed-phase HPLC. A detailed discussion on the development of normal-phase HPLC methods is beyond the scope of this chapter.

Thin-Layer Chromatography (TLC)

TLC is one of the oldest chromatographic methods and is widely used in the pharmaceutical industry. TLC and high-performance TLC (HPTLC) are complementary to reversed-phase HPLC. TLC and HPTLC are typically carried out under normal-phase conditions and therefore can be very useful for separating impurities that cannot be easily separated under reversed phase conditions. One significant advantage of TLC is that detection is carried out on the entire plate following development. This ensures that all of the impurities can be detected whether or not they migrate from the origin, as long as they are separated from the parent and the correct visualization technique is used. Another significant advantage of TLC is the possibility of running multiple samples in parallel rather than running sequentially as is done in HPLC. Some of the disadvantages of TLC include generally decreased sensitivity, resolving power, and the ability to provide accurate quantification as compared to HPLC. For additional details on TLC and HPTLC, see one of the many literature references (12).

Capillary Electrophoresis (CE)

CE is another complementary technique to reversed-phase HPLC. Since there are a significant number of resources available describing CE, a detailed discussion of CE will not be presented. Although a number of detectors are available for CE, the most useful detector for analysis of stress-testing samples is the UV detector. Commercial CE instruments are also available with a PDA detector, which helps when correlating peaks between CE and HPLC. A number of articles have been published describing the use of CE for detection of pharmaceutical impurities (13). A significant amount of work has been done to develop “generic” CE methods that can be used for a wide variety of compounds. For basic solutes, Altria has suggested the use of a phosphate buffer at pH 2.5 (14) and for acidic solutes a borate buffer at a pH of 9.3 (15). Hilhorst has suggested a MEKC strategy for impurity profiling which involves the use of an SDS system and a CTAB system (7a). Analysis of samples on these two systems guarantees, in principle, that all compounds will pass the detector in at least one of the two systems. Altria has also developed a generic MEKC method utilizing lithium dodecyl sulfate and beta cyclodextrin (16). One of the major benefits of CE is that its separation mechanism is different from that of HPLC and it will often give different selectivity. This is illustrated in the analysis of a partially degraded drug sample (Fig. 8). The drug contains two carboxylate groups and is therefore negatively charged under the CE analysis conditions. The top trace is a reversed-phase HPLC chromatogram and the bottom trace is a CE electropherogram. The analysis conditions are given on the figures. The peaks detected using the two techniques were correlated by comparing UV spectra obtained using PDA detectors. Clearly, the two techniques give significantly different selectivities.

Gas Chromatography (GC)

GC is a good choice for analysis of volatile drug substance stress test samples. It is also a complementary technique to HPLC when volatile degradation products are suspected. For example, see the LY297802 example described in chapter 6, where volatile and nonchromophoric degradation products were missed by the HPLC-UV detection scheme but readily detected by extraction and analysis using GC with a flame ionization detector (FID). The FID is usually the GC detector of choice for analysis of stress test samples since it is a nearly universal detector for carbon-containing compounds and has the required sensitivity. The mass spectrometer is another widely used GC detector and can give structural information on the peaks as they elute from the column. One major difference between HPLC and GC is the typical requirement of an internal standard for quantitative analysis using GC.

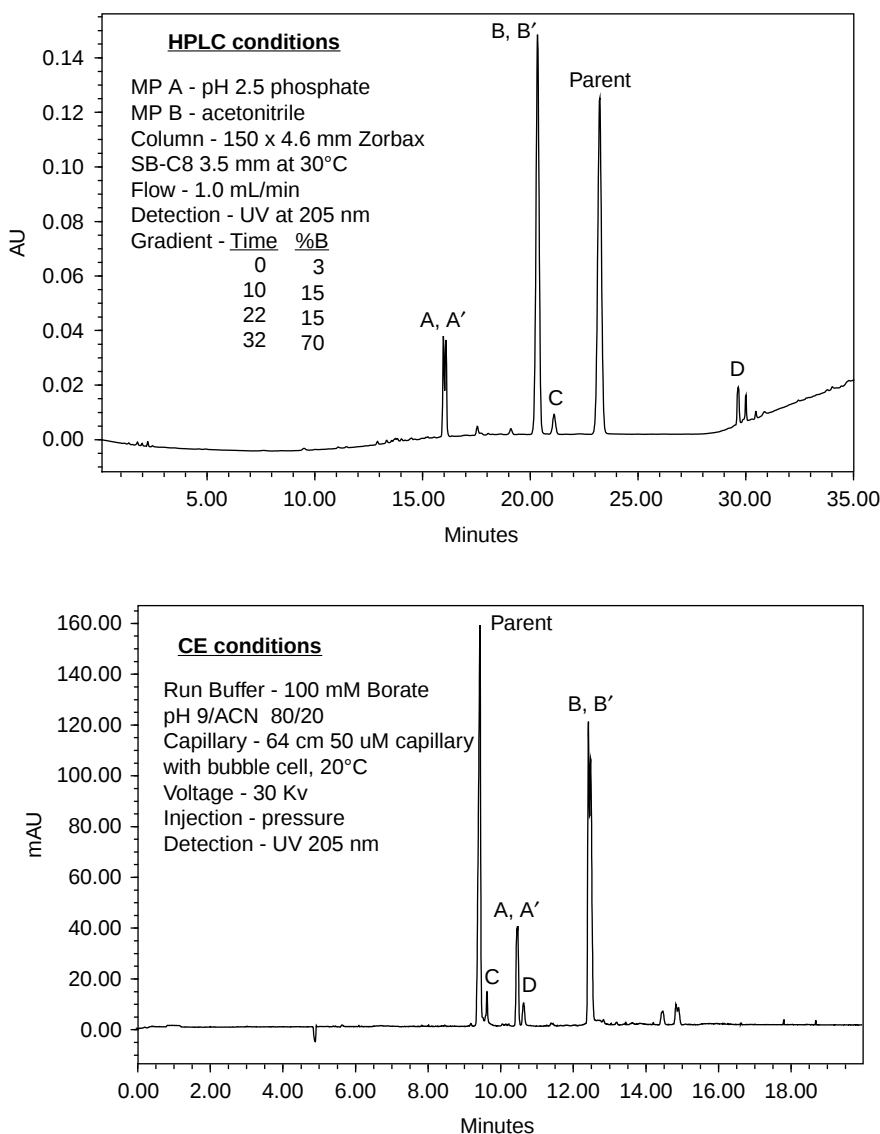


Figure 8 HPLC chromatogram (*top*) and CE electropherogram (*bottom*) obtained on a partially degraded drug sample. Peaks A,A' and B,B' are difficult to separate, diastereomeric pairs.

CONCLUSIONS

Stress testing is an important part of the drug development process as it provides knowledge about the degradation chemistry of drug compounds. This knowledge is used primarily to develop stability-indicating analytical methods but is also useful for other purposes such as formulation development, package development, and the design of official stability studies. Very little formal guidance is available for the design and execution of stress-testing studies and this chapter, along with other chapters in this book, provides some practical guidance on these topics. Typical stress-testing studies involve exposing solid samples of drug substance to heat, heat with humidity, and photostress and solutions or suspensions of the drug substance to hydrolysis conditions at various pH values, oxidative reagents, and photostress. One of the

most important aspects of stress testing is the analysis of stressed samples using a suitable analytical method which, in many cases, is reversed-phase HPLC (or UPLC). This necessitates the development of an HPLC/UPLC method capable of measuring both the loss of the parent compound as well as the levels of degradation products or impurities formed under the stress conditions. General guidance is provided for the development of HPLC/UPLC methods appropriate for analysis of stress-testing samples.

REFERENCES

1. International Conference on Harmonisation, Stability Testing of New Drug Substances and Products, Q1A(R2), February 2003.
2. International Conference on Harmonisation, Photostability Testing of New Drug Substances and Products, Q1B, November 1996.
3. Nelson ED, Thompson GM, Yao Y, Flanagan HM, Harmon PA. Solvent effects on the AIBN forced degradation of cumene: implications for forced degradation practices. *J Pharm Sci* 2009; 98: 959–69.
4. Richter S, Fabris D, Binaschi M. Effects of common buffer systems on drug activity in the case of Clerocidin. *Chem Res Toxicol* 2004; 17: 492–501.
5. International Conference on Harmonisation, Text on Validation of Analytical Procedures, Q2A, October 1994.
- 6a. Argentine M, Owens P, Olsen B. Strategies for the investigation and control of process-related impurities in drug substances. *Adv Drug Delivery Rev* 2007; 59: 12–28.
- 6b. Ahuja S. Assuring quality of drugs by monitoring impurities. *Adv Drug Delivery Rev* 2007; 59: 3–11.
7. Kang J, Wistuba D, Schurig V. Recent progress in enantiomeric separation by capillary electrophoresis. *Electrophoresis* 2002; 23: 4005–21.
8. Biswas K, Castle B, Olsen B. A Simple and efficient approach to reversed-phase HPLC method screening. *J Pharm Biomed Anal* 2009; 49: 692–701.
9. Wren S, Tchelitcheff P. Use of ultra-performance liquid chromatography in pharmaceutical development. *J Chromatography A* 2006; 1119: 140–6.
10. Xue G, Bendick A, Chen R, Sekulic S. Automated peak tracking for comprehensive impurity profiling in orthogonal liquid chromatographic separation using mass spectrometric detection. *J. Chromatography A* 2004; 1050: 159–17110a.
11. Gorecki T, Lynen F, Szucs R, Sandra P. Universal response in liquid chromatography using charged aerosol detection. *Anal Chem* 2006; 78: 3186–92.
- 12a. Gorman PM, Jiang H. Isolation methods I: thin-layer chromatography. In: Ahuja S, Alsante KM, eds. *Handbook of Isolation and Characterization of Impurities in Pharmaceuticals*. Separation Science and Technology, Vol. 5. San Diego, CA: Academic Press, 2003.
- 12b. Sherma J. Planar chromatography. *Anal Chem* 2000; 72: 9–25.
- 13a. Altria KD, Chen AB, Clohs L. Capillary electrophoresis as a routine analytical tool in pharmaceutical analysis. *LCGC* 2001; 19: 972–85.
- 13b. Hillhorst MJ, Derksen AF, Steringa M, Somen GW, DeJong GJ. Towards a general approach for the impurity profiling of drugs by micellar electrokinetic chromatography. *Electrophoresis* 2001; 22: 1337–44.
14. Altria KD, Frake P, Gill I. *J Pharm Biomed Anal* 1995; 13: 951–7.
15. Altria KD, Bryant SM, Hadgett T. Validated capillary electrophoresis method for the analysis of a range of acidic drugs and excipients. *J Pharm Biomed Anal* 1997; 15: 1091–101.
16. Altria KD, McLean R. Development and optimisation of a generic micellar electrokinetic capillary chromatography method to support analysis of a wide range of pharmaceuticals and excipients. *J Pharm Biomed Anal* 1998; 18: 807–13.

5 Stress testing: Relation to the development timeline

Steven W. Baertschi, Bernard A. Olsen, Karen M. Alsante, and Robert A. Reed

As has been discussed elsewhere (1,2), for a novel drug candidate that progresses from discovery through preclinical and clinical stages of development and eventually to the market, stress testing is not a “one-time” event. Instead, stress testing is typically performed at several stages in the “life cycle” of a novel drug candidate with different goals (and therefore often different strategies and levels of thoroughness) depending on the stage of development. For example, stress testing of a solid drug substance requires the testing of material that is representative, that is, the material that will be used during clinical trials or the material that will be marketed. Thus, the drug substance should be the same solid form (e.g., same salt, solvation state, and polymorphic form) with similar solid characteristics (e.g., particle size, surface area, crystallinity, etc.). Since early lots of a drug candidate are generally obtained from synthetic routes and processes that will not be the same as that used for the final marketed form, solid-state stress testing of early lots may not accurately reflect potential active pharmaceutical ingredient (API) stability issues with new routes and processes. Therefore, API stability needs to be evaluated over the course of development. This is especially critical to reassess solid-state stress testing as a result of API process changes (i.e., synthetic steps, crystallization solvents, milling/micronization, etc.) and changes in polymorph/salt form.

Similarly, it is becoming more and more common to design simple formulations (e.g., powder in a capsule, technology platform formulations) that require minimal resource investment in process development and formulation design prior to proof-of-concept (i.e., Phase IIA) of the drug candidate in patients. Once proof-of-concept has been established, then process development and formulation design focus changes toward the development of a commercial process and product. The evolution of formulation composition, processing, finishing (e.g., film coating) and packaging all lend to require that the applicability of the early forced stress testing for the drug product be revisited throughout the remainder of the development phase since each new excipient can potentially react with the API yielding new degradation products to monitor.

A significant factor in the design of stress-testing studies at different stages is the reality that only a small percentage of novel drug candidates make it to the market—the vast majority fails at some point during the drug development process due to factors such as unexpected toxicity, poor absorption, or bioavailability (or other biopharmaceutical or metabolism problems), or lack of efficacy (3,4). Since there is a high attrition rate (approximately 90% of compounds entering development will fail), it is not cost-effective to perform the kind of thorough research needed for a marketed product for every new drug candidate. As an outcome of the need for increased efficiencies of resource use in early stage drug development, *in silico* tools for predicting degradation routes and species are becoming more popular (see chaps. 2, 19, 20, for more details). However, capturing this knowledge is key to applying to other drug candidates with similar structures to prevent duplication of effort and recognize resource savings at the early stage. Furthermore, recent emphasis on the potential formation of genotoxic degradation products has—in addition to impact synthetic route selection and salt selection—also increased the use of *in silico* evaluations. Thus, stress testing is worth discussing in the context of typical life cycles of a novel drug candidate: (i) drug discovery, (ii) preclinical/early phase, (iii) “commercialization” or late phase, and (iv) line-extensions and products on the market.

DRUG DISCOVERY STAGE (STRUCTURE–ACTIVITY RELATIONSHIP AND COMPOUND SELECTION STAGE)

During drug discovery, compounds with the desired biological activity (ies) are identified and structure–activity relationships are determined. In addition to biological activity, compounds need to have appropriate biopharmaceutical properties (3,5), stability being one of the critical

properties (6). The goal of stress testing or stability studies at this stage is to determine whether or not a compound has stability sufficient for the desired routes of administration and would be able to have a reasonable shelf life as a marketed product. Such stress testing studies are typically very short in duration (7) and are limited in scope (with an emphasis on high throughput), analytical methodologies are typically generic (i.e., not specifically designed for the individual compound) and therefore are usually less thoroughly challenged and less rugged than would be expected for regulatory submissions. The goal of these studies is to provide rapid guidance to discovery efforts geared toward designing compounds with the desired biological activities and biopharmaceutical properties (e.g. solubility, permeability, and stability). Degradation prediction analyses from software programs such as Zeneth (8) (a program designed specifically for prediction of potential chemical degradation pathways under pharmaceutically-relevant conditions) as well as older software packages (e.g., CAMEO, CambridgeSoft, Cambridge, MA) (9) are useful at this stage when the resources are not available for time-consuming experimental degradation studies (see chap. 20). Also useful at this stage is knowledge gained from previous studies on compounds with similar structures. Database storage and retrieval of such information can be valuable in focusing efforts on the most critical degradation experiments (10). In addition, communications with discovery scientists can play a critical role. For example, predicted degradation products for a candidate may be available as samples from discovery/drug metabolism research. Such degradation samples can be run as standards during early method development efforts to provide degradation information when time cannot be invested in LC/MS structural elucidation studies. At this stage of development, potential drug candidates can be ranked in context of types of degradation (e.g., hydrolytic, oxidative, etc.) with less emphasis on structural elucidation of degradation products. However, it is critical to capture as much learning as possible on candidates since the majority will have a short life cycle. Any learning on degradation (e.g., degradation conditions where the compound demonstrates instability, mechanistic and/or structural data) can be applied to future candidates in the same class of compounds or with similar structure, etc. Moreover, attempts to mine long-term stability data from structural analogs that have progressed further into late stage development can also strengthen the predictability of a short-term stress test for long-term storage (another benefit of having a drug degradation database as described above) (11).

PRECLINICAL/EARLY PHASE (PRECLINICAL TO PHASES I/II)

Once a compound with the desired biological activities and biopharmaceutical properties has been identified and selected for clinical evaluation, information about the stability of the compound needs to be gathered in a more rigorous process. At this stage, the primary goals of stress testing are to develop valid stability-indicating analytical methods that are specifically developed for the compound being evaluated. These methods must be stability indicating (i.e., able to separate the analyte from the potential degradation products and impurities that are specific to the compound and synthetic route being evaluated). In some cases, highly resolving generic methods have also been applied at this stage, which may provide the needed selectivity for a variety of compounds (see chap. 4 for more detail on this approach).

The goal of these studies should be to demonstrate reasonable assurance that the stability of the compound can be maintained throughout the clinical trial period. In addition, stress-testing studies should provide basic stability information to help early formulation development. Generally, identification of degradation products observed during stress testing is not critical during this stage, although there are many times when such information can be very useful to the further development of the compound; typically, structural information at this stage is limited to data obtained through LC/MS analyses (e.g., molecular weight, fragmentation, etc.).

It is worth mentioning that there are various philosophies in the pharmaceutical industry regarding the amount of stress testing (and development work in general) that is needed during the various phases of preclinical and clinical development. The differences in approaches to

stress testing are documented by Alsante et al. in a benchmarking study of current stress-testing practices in 20 pharmaceutical companies (12) and are also discussed more thoroughly in this text (chap. 2). Note also that this survey was published in 2003 and advances in stress testing best practices have occurred since then, as documented in the first edition of this book and in chapter 2 in the current edition of this book.

Most companies (out of 20 surveyed, Fig. 1) first perform degradation studies in the pre-clinical phase of development. Five companies out of 20 first perform stress testing in the discovery stage. Most companies conduct additional stress studies as the clinical trials progress by Phases 1 through 3. The practice of repeating or conducting additional stress-testing studies varies by stage of development. Repeating or conducting additional stress-testing studies is typically associated with factors such as analytical methodology improvements, changes in the drug substance (e.g., changes in salt or polymorph, solid-state physical changes, etc.), or changes in the drug product formulation as the compound progresses through the development process. Of those companies that repeat or conduct additional stress-testing studies, eight reported doing so in more than one phase.

For the drug product, 18 out of 20 companies perform stress-testing studies (Fig. 2). Companies first perform these studies between discovery and Phase II but mostly in preclinical.

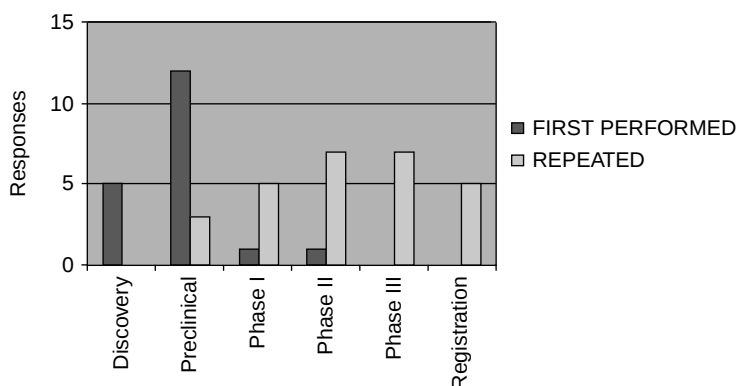


Figure 1 Drug substance stress testing studies performed by phase of development ($n = 19$ first performed, $n = 17$ repeated) [see Ref. 12].

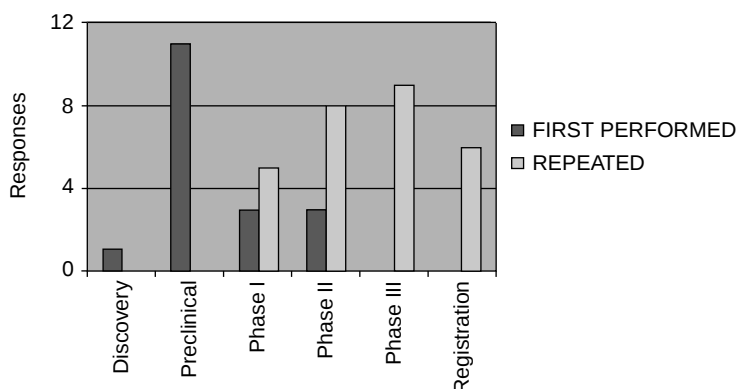


Figure 2 Drug product stress testing studies performed by phase of development ($n = 18$ first performed, $n = 16$ repeated) [see Ref. 12].

Phases in which the studies are repeated vary across companies from Phase I through registration. Of those that repeat drug product stress-testing studies, eight companies reported doing so in more than one phase.

“COMMERCIALIZATION” STAGE OR LATE-PHASE DEVELOPMENT (PHASE II/III TO REGULATORY SUBMISSION)

Once a drug candidate has shown an acceptable safety profile and has also shown appropriate efficacy (e.g., during Phase II clinical trials), larger-scale clinical trials (i.e., Phase III) are warranted. At this stage of development, it is apparent that the regulatory authorities expect the pharmaceutical companies to do the needed research to fully characterize and understand the drug compound. The FDA has published a guidance for industry for Phase 2 and Phase 3 studies conducted under INDs (13). Quoting from the guidance for Phase 2 (drug substance) studies:

Performance of stability stress studies with the drug substance early in drug development is encouraged, as these studies provide information crucial to the selection of stability indicating analytical procedures for real time studies.

It is noteworthy that stress testing is not mentioned for the drug product for Phase 2 studies. With regard to Phase 3, the guidance indicates the following (for the drug substance):

If not performed earlier, stress studies should be conducted during Phase 3 to demonstrate the inherent stability of the drug substance, potential degradation pathways, and the capability and suitability of the proposed analytical procedures. The stress studies should assess the stability of the drug substance in different pH solutions, in the presence of oxygen and light, and at elevated temperatures and humidity levels. These one-time stress studies on a single batch are not considered part of the formal stability program. The results should be summarized and submitted in an annual report.

With regard to the drug product studies during Phase 3, the guidance indicates the following:

For certain drug products, one-time stress testing can be warranted to assess the potential for changes in the physical (e.g., phase separation, precipitation, aggregation, changes in particle size distribution) and/or chemical characteristics (e.g., degradation and/or interaction of components) of the drug product. The studies could include testing to assess the effect of high temperature, humidity, oxidation, photolysis, and/or thermal cycling. The relevant data should be provided in an annual report.

The goals of stress testing at this stage go beyond ensuring stability during the clinical trials since the intention is to bring the product to the market. The goals are therefore to understand all potential stability issues including storage, distribution, short-term temperature excursions, and formulation issues (and even potential patient “in-use” stability issues), as well as to provide a thorough foundation for validation of stability-indicating analytical methods for the marketed life of the compound. A thorough understanding of potential degradation products and pathways (including mass balance understanding) should be developed during this stage, keeping in mind that this information will form “an integral part of the information provided to regulatory authorities” in the marketing authorization submission. ICH Guidance Q3A and Q3B (14) are typically fully applied at this stage of development, and require that all degradation products in the drug substance and the

formulation product that exceed the total daily intake (TDI) “identification thresholds” be fully characterized. It is worth noting here that any degradation products for which structures have been elucidated should be assessed for genotoxic potential, per the EMEA guidance on genotoxic impurities (15) and the FDA draft guidance (16). The topic of genotoxic impurities is currently being developed by ICH (17). For a more thorough discussion of this topic, see chapter 19. More recently, a PhRMA “white paper” on this topic is also under preparation (18) and targeted for publication in the near future.

LINE-EXTENSIONS (NEW FORMULATIONS, NEW DOSAGE FORMS, NEW DOSAGE STRENGTHS, ETC.), OLDER PRODUCTS ALREADY ON THE MARKET (UPDATING METHODS, ASSESSING PROCESS CHANGES) GENERIC DRUGS

After registration, changes to the drug substance or drug product manufacturing process are often desired to reduce cost, increase quality or reliability, or reduce environmental impact. Manufacturing site and scale changes are also common. Risk-based guidances (19) are often used to assess the significance of a process or formulation change, and may require stability studies to be conducted to demonstrate that the proposed changes do not adversely impact the already established stability characteristics of the product. The stability assessment is usually desired in a much shorter period of time than that required for long-term studies or even the 3–6 months required for accelerated conditions. A rapid stability assessment is also needed for line-extensions involving new formulations or different strengths of an existing product. In chapter 18, the use of “highly accelerated” conditions is described for comparative stability studies or for developing stability models useful for a broad range of conditions. In this mode, elevated temperatures and/or humidities beyond the ICH accelerated stability conditions are used to compare the stabilities of products made in different ways or to develop predictive models. Such highly accelerated or stress studies can be useful in evaluating process changes where a baseline of knowledge about the stability characteristics of the compound already exists. Information about the stability of new formulations of existing active components can also be obtained quickly using highly accelerated conditions. These studies may reveal stability issues much more rapidly than traditional methods and lead to more efficient and effective drug development.

Another important consideration during the lifecycle of a drug is the development of new dosage strengths, new dosage forms, new formulations, and alternate routes of administration. Each new development will require new or modified stress testing and stability studies, as it cannot be assumed that degradation rates and pathways will remain the same as those in the original product. New or modified analytical methodologies may also be required, and therefore new or revised stress testing studies will need to be performed as part of the analytical method development process. New or modified analytical methodologies can also lead to the discovery of new impurities (in line-extensions and even in existing products) that were not detected with previous methods, a significant challenge for pharmaceutical companies.

At the time of patent expiry, data on forced stress studies is typically limited, that is, either not published or available through freedom of information. Additionally, the compendia (e.g., USP, PhEur or JP) often do not have methods established, and if they do, monograph methods may indeed be (and are supposed to be) stability indicating; however the information in the method may not necessarily be sufficient to discern this. Therefore, noninnovator companies will likely need to conduct their own set of forced stress studies to establish (i) a thorough understanding of potential degradation products for the API and drug product, (ii) demonstrate for the new source of API or drug product that the synthetic pathway or process (for API) and formulation and process (for the drug product) can be adequately characterized with appropriate test methods, and (iii) guide the development and scale up for the API and drug product manufacture.

CONCLUSION

Developing a thorough understanding of potential degradation products for API and drug product for a new chemical entity is a continuous process that initiates early in drug discovery, and continues through process and formulation change for the lifetime of a drug. Innovator companies often build a solid knowledge base for the potential degradation chemistries by leveraging the experience across multiple product development phases, and organizations within the company that perform these studies. Clearly, how resources are leveraged to accomplish this needed understanding is not consistent across companies, likely due to how risk and resource investment models are established within each company. Finally, regulatory guidance has established critical milestones to achieve given levels of understanding, but have left the path, and associated timelines, to achieve these goals up to individual companies.

REFERENCES

1. Zelesky T, Alsante KM, Coutant M. Degradation and impurity analysis for pharmaceutical drug candidates. In: Ahuja S, Alsante KM, eds. *Handbook of Modern Pharmaceutical Analysis*, 2nd edn. New York: Academic Press, 2010.
2. Reynolds DW, Facchine KL, Mullaney JF. Available guidance and best practices for conducting forced degradation studies. *Pharm Tech* 2002; 26: 48–54.
3. Lipper RA. How can we optimize selection of drug development candidates from many compounds at the discovery stage? *Mod Drug Discov* 1999; 2:55–60.
4. Prentis RA, Lis Y, Walker SR. Pharmaceutical innovation by the seven U.K. owned pharmaceutical companies. *Br J Clin Pharmacol* 1985; 25: 387–96.
5. Palucki M, Higgins JD, Kwong E, Templeton AC. Strategies at the interface of drug discovery and development: early optimization of the solid-state phase and preclinical toxicology formulation for potential drug candidates. *J Med Chem* 2010; 53: 5897–905.
6. Di L, Kerns, EH. Stability challenges in drug discovery. *Chem Biodiv* 2009; 6: 1875–86.
7. MacFaul PA, Ruston L, Wood JM. Activation Energies for the Decomposition of Pharmaceuticals and Their Application to Predicting Hydrolytic Stability in Drug Discovery. *Med Chem Comm* 2011; 2: 140–2.
8. For more information about Zeneth, see <https://www.lhasalimited.org/>, Lhasa Limited, Leeds, UK.
9. Jorgensen WL, Laird ER, Gushurst AJ. CAMEO: a program for the logical prediction of the products of organic reactions. *Pure Appl Chem* 1990; 62: 1921–32.
10. Alsante KM, Snyder KD, Swartz M, Parks C. Application development using out-of-the-box-software: a structure searchable degradation/impurity database. *Sci Comp Instrum* 2002; 30–7.
11. Harmon PA, Kosuda K, Nelson E, Mowery M, Reed RA. A novel peroxy radical-based oxidative stressing system for predicting oxidative instability of active pharmaceutical ingredients. *J Pharm Sci* 2006; 95: 2018.
12. Alsante KM, Martin L, Baertschi SW. A stress testing benchmarking study. *Pharm Technol* 2003; 27: 60–72.
13. FDA, INDs for phase II and III studies of drugs, including specified therapeutic biotechnology derived products. *Federal Register (Notices)* 1999; 64: 19543–4.
14. (a) International Conference on Harmonization Guidance to Industry, Q3A (R2) Impurities in New Drug Substances, June 2008; (b) International Conference on Harmonization Guidance to Industry, Q3B (R2) Impurities in New Drug Products, June 2006.
15. Committee for Medicinal Products for Human Use (CHMP). Guideline on the Limits of Genotoxic Impurities. EMEA/CHMP/QWP/251344/2006.
16. FDA. Guidance for Industry: Genotoxic and Carcinogenic Impurities in Drug Substances and Products: Recommended Approaches, 1 January, 2008.
17. International Conference on Harmonization, Final Concept Paper M7: Genotoxic Impurities, dated November 27, 2009. Endorsed by the ICH Steering Committee on 9 June 2010.
18. Baertschi SW, Elder D, Kleinman M. Strategies for addressing potentially genotoxic degradants in Active Pharmaceutical Ingredients and formulated product: a PhRMA white paper. Draft paper by the PhRMA Limited Duration Key Initiative Team.
19. (a) Guidance for Industry, SUPAC IR—Immediate Release Solid Oral Dosage Forms Scale-Up and Postapproval Changes: Chemistry, Manufacturing, and Controls, *In Vitro* Dissolution Testing, and

In Vivo Bioequivalence Documentation, Center for Drug Evaluation and Research, 1995; (b) Guidance for Industry, SUPAC MR—Modified Release Solid Oral Dosage Forms Scale-Up and Postapproval Changes: Chemistry, Manufacturing, and Controls, *In Vitro* Dissolution Testing, and *In Vivo* Bioequivalence Documentation, Center for Drug Evaluation and Research, 1997; (c) Guidance for Industry, SUPAC-SS—Nonsterile Semisolid Dosage Forms Scale-Up and Postapproval Changes: Chemistry, Manufacturing, and Controls, *In Vitro* Dissolution Testing, and *In Vivo* Bioequivalence Documentation, Center for Drug Evaluation and Research, 1997.

6 | Oxidative susceptibility testing

Paul Harmon and Giovanni Boccardi

INTRODUCTION

Water and molecular oxygen (dioxygen) are the two ubiquitous molecules that most frequently affect the stability of a drug substance. Though acids and bases are the main catalysts that control the hydrolytic behavior of organic compounds, they are not the principal factors in oxidations. In many cases, oxidation involving dioxygen is hard to understand and may also seem difficult to reproduce. This difficulty is compounded by consideration of dioxygen as a potential reactant with organic substrates. In the orbital diagram of molecular oxygen, the highest occupied molecular orbitals are two degenerate π^* orbitals in which there must be two electrons (Fig. 1). The ground state, according to the Hund rule, is the state in which these orbitals are occupied by one electron and the spins are parallel: this is the triplet ground state ($^3\Sigma_g^-$) of atmospheric molecular oxygen (Fig. 1). However, the vast majority of organic molecules are in the singlet state, and the reaction:



is spin-forbidden. For this reason, a large number of organic molecules, in spite of the large negative value of the Gibbs free energy of oxidation, are kinetically inert toward dioxygen.

How then does oxidation of drug molecules in formulated solid dosage forms proceed? A significant clue is the common experience of many pharmaceutical scientists, in which a pure drug substance is quite stable toward oxidation, but in the formulated drug product the drug substance oxidatively degrades. One significant contributor to this effect is the role that excipient impurities often play as “initiators” of oxidation (1–4). As will be described below, these impurities can give rise to peroxy and alkoxy radicals $R_{imp}OO\bullet$ and $R_{imp}O\bullet$ which are actually the species that initially react with drug molecule carbon–hydrogen (D–H) bonds. These reactions produce drug molecule radicals ($D\bullet$) which by virtue of their unpaired electron are in a triplet state, and thus are very reactive with dioxygen. This simple concept explains how the spin forbidden reaction (1) is readily obviated and accounts for variable formulation and batch-dependent oxidation rates often encountered by pharmaceutical scientists.

The aim of this chapter then is to provide the reader with the necessary theoretical framework to understand most oxidations one is likely to encounter and to review the current methodologies, tests, and accepted practices to predict to what extent drug molecules will be oxidatively sensitive. The first edition of *Pharmaceutical Stress Testing* (5) did not consider several more recently described methodologies aimed at examining oxidative liability of drug molecules. These include methodologies based on DSC (6), cyclic voltammetry (7,8) and a novel type of peroxy radical-based stressing system (9). While the latter two of these will be discussed in some detail, an additional focus of this chapter will be to incorporate more recent work on practical experimental aspects such as oxygenation requirements, solvent choice and pH effects for the two most common oxidative susceptibility tests—azobitrile initiation and the hydrogen peroxide test. The goal of these methodology optimizations is to ensure that drug substances are exposed to the appropriate oxidants during the stress test.

In section “Mechanistic Background for the Most Common Oxidation Routes,” we will review the mechanistic background for three oxidation routes. The first is autoxidation, by far the most common oxidative route, which is “initiated” as described above and involves a radical chain process. The second route is oxidation of drug molecules by organic hydroperoxides and/or hydrogen peroxide in a nonradical reaction, in which all electrons are paired. Finally, oxidation mediated by single electron transfer (SET) to dioxygen will be considered. In section “Practical Tests and Considerations for Oxidative Susceptibility Testing,” we will review practical experimental aspects of solution phase oxidative susceptibility testing. Finally, in section “Summary and General Strategy of Oxidative Susceptibility Testing,” we will provide a brief overall strategy of oxidative susceptibility testing.



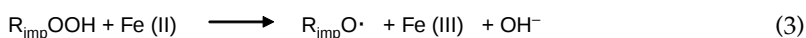
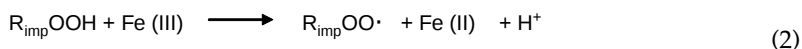
Figure 1 Electronic configurations of molecular oxygen: triplet ground state and the singlet first excited state. The figure shows the highest occupied molecular orbitals.

MECHANISTIC BACKGROUND FOR THE MOST COMMON OXIDATION ROUTES

Autoxidation

Autoxidation Mechanism

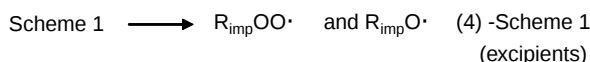
The mechanism of autoxidation has been studied extensively in solution and reviewed (1,2,10). While some minor differences between solution and solid state (oral dosage form) autoxidation might be expected, the solution-based understanding of autoxidation provides the basis for describing autoxidation in the solid state. A brief overview follows. Autoxidation is described in three stages: initiation, propagation, and termination. In the current context, we chose to specifically highlight the role that excipient impurities can play in the initiation stage of autoxidation in solid dosage forms. By far, the most common excipient impurities which are capable of initiating oxidation are organic hydroperoxides ($R_{\text{imp}}\text{OOH}$). Organic hydroperoxides are found at trace levels (hundreds to thousands of nanomoles/g excipient) in numerous excipients such as Tween 80, PEG 400, HPMC, PVP, and others, and have been well studied (11–13). Hydroperoxides can be catalytically degraded by trace levels of transition metal ions (in particular Fe(III), which is ubiquitous) to form equimolar amounts of peroxy and alkoxy radicals as shown in Scheme 1:



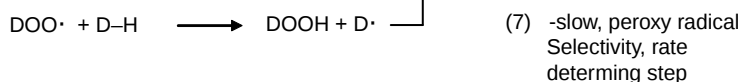
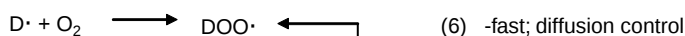
Scheme 1 Catalytic cycle for Fe(III)/(II) and an excipient-related hydroperoxide $R_{\text{imp}}\text{OOH}$.

Given this initiation step, the subsequent propagation and termination steps for autoxidation of hydrogen bearing sp^3 carbon atoms of a drug molecule (D-H) is shown in Scheme 2:

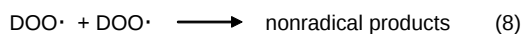
Initiation



Propagation



Termination



Scheme 2 Autoxidation mediated by drug-based peroxy radical ($\text{DOO}\cdot$).

The initiation of oxidation of the drug molecule is shown in Eqs. (5a) and (5b) as abstraction of drug H-atoms from D-H bonds by the impurity-derived radicals. Both Eqs. (5a) and (5b) generate D• radicals. Given the known large rate constant for oxygen reaction with carbon radicals, which is near $10^9 \text{ M}^{-1}\text{s}^{-1}$ or higher at 300 K (14) and the general availability of dissolved oxygen in solution or oxygen gas in the case of a solid dosage form, Eq. (6) shows that the primary reaction expected is formation of drug-derived peroxy radicals DOO•. Equation (7) is the key reaction in autoxidation, the reaction of the drug-derived peroxy radical with the drug itself. Peroxy radical reactions are relatively slow [for example, the cumene rate constant k_p is $0.18 \text{ M}^{-1}\text{s}^{-1}$ at 303 K (10)] and are thus typically the rate-limiting step in Scheme 2. Both products in Eq. (7) are important. The first, DOOH, is the drug hydroperoxide and represents the first stable or metastable oxidation product one might identify either in a solution or an oral dosage form. The second product in Eq. (7) is another drug radical D•, which again will oxygenate (upward arrow in Scheme 2) to give another drug peroxy radical as shown in Eq. (6). This repeating process [Eq. (6) to Eq. (7) to Eq. (6), etc.] is referred to as peroxy radical chain propagation or just propagation. For “oxidatively sensitive” drugs, the amount of drug oxidized in Eqs. (6) and (7) can be expected to be significantly larger than the drug oxidized in the initiation steps Eqs. (5a) and (5b). Finally, the termination reaction in Eq. (8) occurs when the D-H concentration begins to diminish enough that drug peroxy radicals encounter each other and react to give non-radical products [mechanism is detailed below in Eq. (9)]. In an oral dosage form, the D-H concentration decrease could be viewed as a local phenomenon such as degradation in a defect zone or in an amorphous region of an otherwise crystalline lattice.

Some further discussion on the selectivity of the key peroxy radical reaction in Eq. (7) is warranted. Peroxy radical C-H bond reaction rates are related to the substrate's C-H bond dissociation energy in comparison to that of the peroxy radical ROO-H bond energy, which is about 89 kcal/mole (1,2,10). Thus substrate C-H bonds with bond energies less than 89 kcal/mole may react relatively rapidly with peroxy radicals while higher bond energy C-H bonds will react much slower. Compilations of C-H bond energies and reaction rates with peroxy radicals are available (15) and can be useful in understanding potential C-H bond reactive sites. Most C-H bond energies are significantly larger than 89 kcal/mole which accounts for the selectivity of peroxy radical reactions. In addition, peroxy radical may also undergo addition reactions with olefin bonds (not shown in Scheme 2) which will have all the same mechanistic outcomes as shown in Scheme 2. Many drug molecules will have only very slow (if any) reaction with peroxy radicals, while others may have significantly faster reaction rates. This difference, in the context of Scheme 2, is what makes one drug substance “oxidatively stable” while another is “oxidatively sensitive” given similar initiation rates in Eqs. (5a) and (5b).

We will not go in depth into the subject of antioxidants used in pharmaceutical formulations (for reviews, see Refs. 1-3,16). It is worthwhile however to note from Scheme 2 why the autoxidation mechanism can often be effectively inhibited by phenolic antioxidants such as butylated hydroxyl anisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate. These compounds have two unique properties: (i) very low energy O-H bonds which can rapidly donate hydrogen atoms to the propagating peroxy radicals in Eqs. (6), (7), and (ii) the resulting antioxidant radicals are not reactive with molecular oxygen themselves, being stabilized through delocalization and steric hindrance (16). These “chain breaking antioxidants” work particularly well if the effective peroxy radical chain propagation length is long since “quenching” of one peroxy radical in this way dramatically reduces the amount of drug subsequently oxidized. The fact that BHA and BHT are commonly and successfully used to stop oxidation in oral dosage forms suggests that even in the solid state, propagation chain lengths are significantly greater than one.

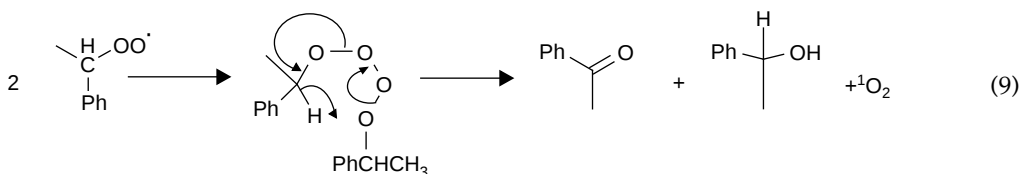
This chapter does not deal with the kinetics of oxidative degradation, treated elsewhere in detail (17), because the authors believe degradation kinetics is a more advanced task in drug development than the oxidative susceptibility stress test. One aspect of the kinetic treatment of Scheme 2 should be highlighted, however. Expressions for the rate of autoxidation in solution are proportional to the product of the substrate concentration and the rate constant of the

peroxy radical hydrogen atom abstraction [Eq. (7), Scheme 2]. Typically, there is no observed rate dependence on dissolved oxygen concentrations for solutions in equilibrium with ambient oxygen (0.21 atmospheres) given that the rate constant for Eq. (6) is much larger than the rate constants for Eq. (7) in Scheme 2 (18,19). Oxygenation will be briefly revisited in section “The Fate of the Unstable Peroxy Species: The Origin of the Stable Degradation Impurities.”

The Fate of the Unstable Peroxy Species: The Origin of the Stable Degradation Impurities

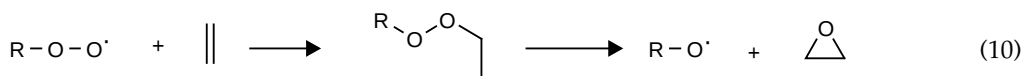
The degradation profile from Scheme 2 will reflect the peroxy radical chemistry in Eqs. (6)–(8). Hydroperoxides are the primary degradation products of autoxidation and can be found as degradation impurities, but the most stable products develop in side reactions involving hydroperoxides and peroxy radicals. Some of these processes surrounding the formation and decomposition of hydroperoxides will be summarized here.

- *Termination reactions.* A very common termination reaction, known as the Russell mechanism from its discoverer, is the recombination of two peroxy radicals to form an unstable tetroxide that decomposes through a concerted mechanism to yield an alcohol moiety and a carbonyl (20):



The oxygen molecule produced by this mechanism is in the singlet state. Equation (9) is shown for a secondary benzylic peroxy radical, but is general for primary and secondary peroxy radicals.

- *Epoxide formation.* Peroxy radicals can react with carbon–carbon double bonds to produce epoxides (21):



This reaction introduces the concept of co-oxidation in that the alkoxy radical $\text{RO}^\bullet$ produced in Eq. (10) may also oxidize substrates in addition to peroxy radicals.

- *Acid decomposition.* Hydroperoxides are decomposed by acids (Fig. 2). The first step is protonation, and two paths are possible for the protonated hydroperoxide. The first is elimination of a water molecule, giving the oxonium ion(I) that can rearrange, yielding an alcohol and a ketone. This path is favored by substituents on the carbon atoms that can migrate. A well-known example is cumene oxidation, which is the basis of the industrial phenol-acetone process. The second path is the elimination of hydrogen peroxide to yield the carbocation(II) in Figure 2. The carbocation can add a nucleophile such as water or an alcohol, or other carbocation scavengers. Carbocations can also rearrange causing ring expansion, or elimination of a proton to give a carbon–carbon double bond (22).

Oxidation by Organic Hydroperoxides and Hydroperoxide

Hydroperoxides ROOH are the first metastable product formed in autoxidation as shown in Scheme 2. Hydrogen peroxide can be produced by elimination during acid decomposition of hydroperoxides as shown in Figure 2. Thus, reaction of drug molecule functional groups with hydroperoxides needs to be explicitly considered and this represents a distinct oxidation

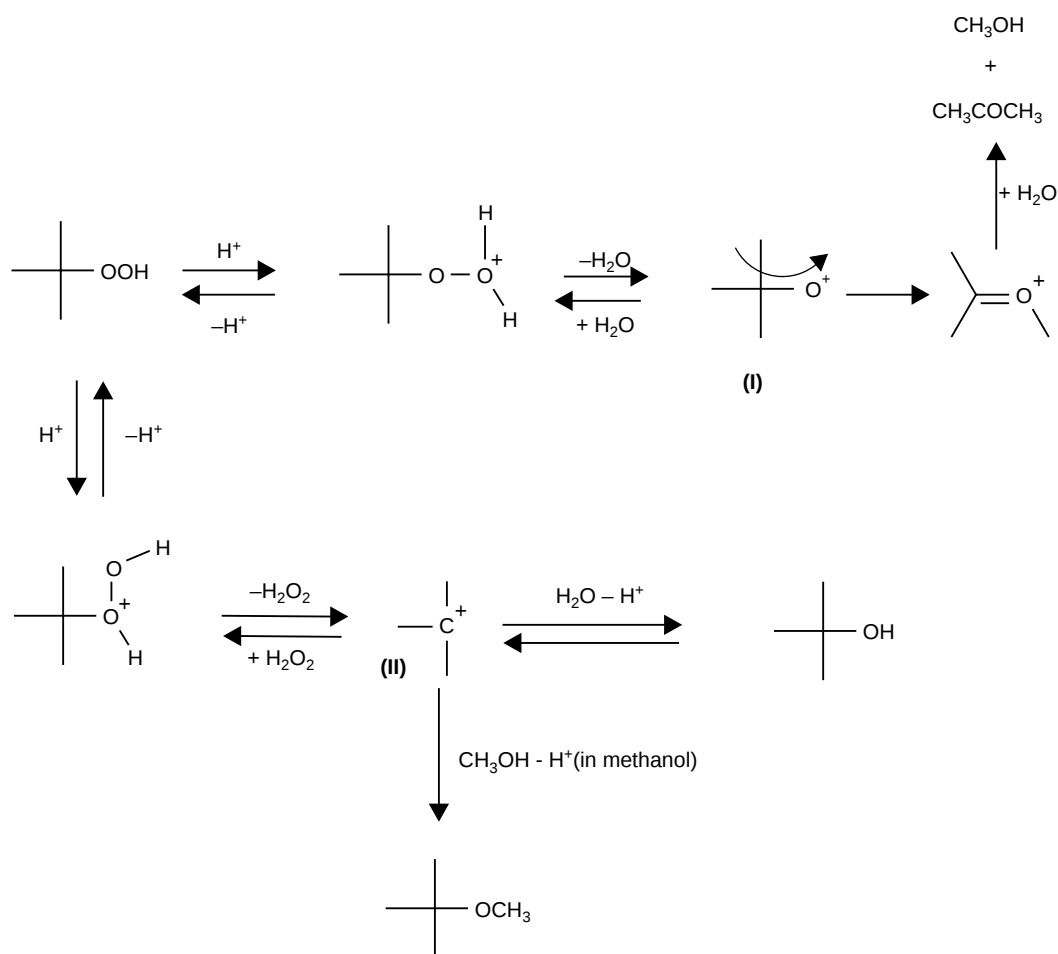
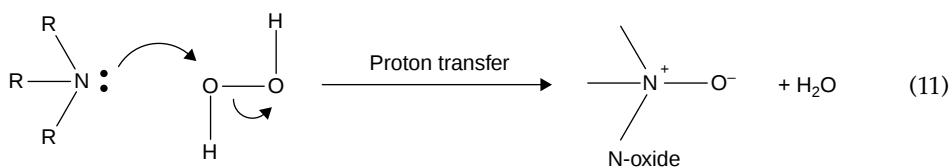
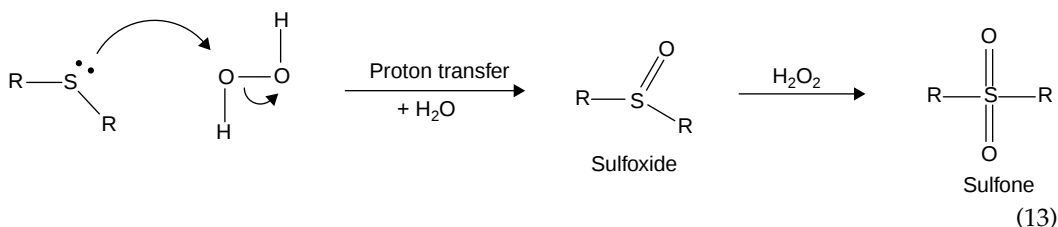
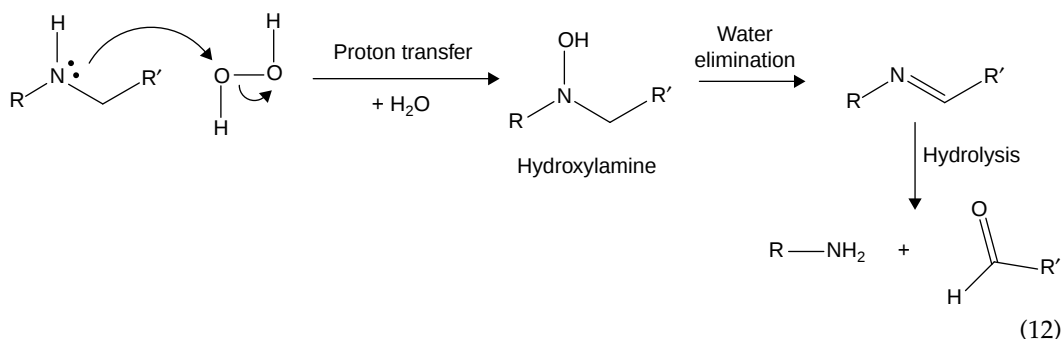


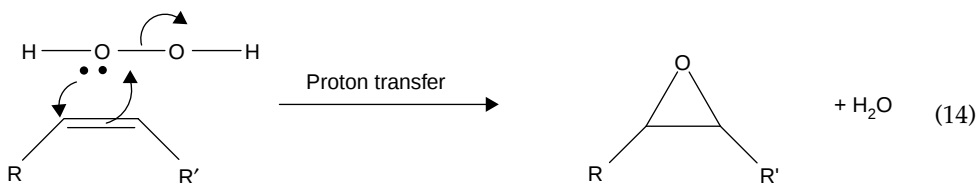
Figure 2 Acid decomposition of hydroperoxides (22): main pathways. Dehydration and rearrangement (*upper*), loss of H_2O_2 , and nucleophilic attack of the solvent (*bottom*).

pathway from autoxidation. In this context, we are interested in reactions which may occur under long-term storage conditions; that is, at a maximum of 30°C or 40°C. Equations (11)–(14) show the most common reactive groups of drug molecules. Considering the intact hydroperoxide as the oxidizing reagent, Eqs. (11) and (12) show electrophilic attack on tertiary and secondary amines to form the *N*-oxide and hydroxylamine, respectively. The tertiary amine reaction is generally more favorable. Equation (12) shows further decomposition of the hydroxylamine for completeness. Note that the amine reactions will have a marked pH dependence in Eqs. (11) and (12); the reactions being much slower in the protonated state. Equation (13) shows electrophilic attack on a thioether to give a sulfoxide and then further a sulfone:



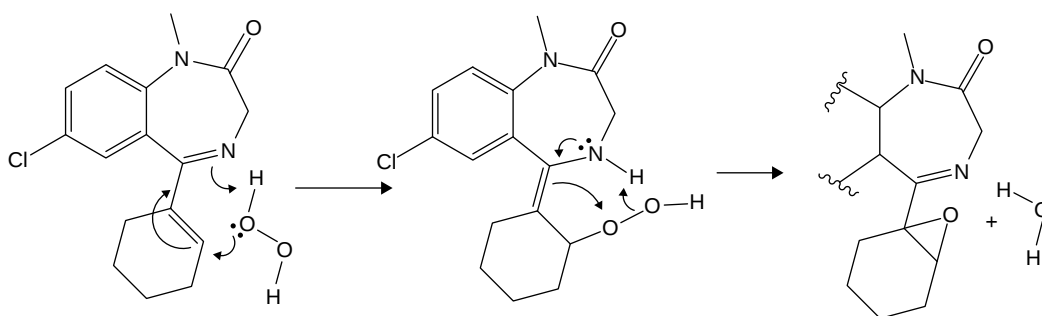


Equation (14) shows the epoxidation of a carbon–carbon double bond. This reaction is typically much slower than Eqs. (11)–(13) and is more appropriately viewed as a nucleophilic attack of the hydroperoxide on the olefin bond. In Eqs. (11)–(14), all the reactions are “ionic” in that all electrons are paired, radicals are not involved:

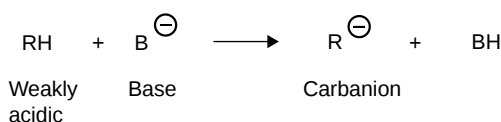
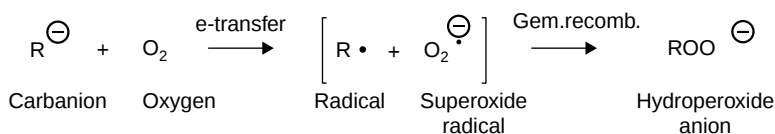


The epoxidation reaction is worthy of further discussion. Under the mild conditions described here and discussed further below in section “Practical Tests and Considerations for Oxidative Susceptibility Testing,” reaction (14) proceeds at a reasonable rate only if there is some sort of stabilizing/supporting structural effects associated with the R and R' groups which facilitate the attack of the relatively weak nucleophile (hydrogen peroxide). Tetrazepam offers a likely case example of this type of effect, where the tetrazepam epoxide is obtained in fairly high yield from dilute hydrogen peroxide solution in methanol or acetonitrile at 40°C (23). Figure 3 shows how tetrazepam likely facilitates the nucleophilic attack and epoxide formation.

While more reactive reagents such as peracetic acid and *m*-chloroperbenzoic acid could be used to generate epoxides for HPLC selectivity or other purposes, the goal of the stress test in the current context is to expose drug substances only to the hydroperoxide oxidants that might realistically be encountered in solid dosage forms. In this way, structural enhancements to reactivity such as that suggested in Figure 3 are uniquely revealed and recognized.



Tetrazepam

Figure 3 Likely reaction mechanism of tetrazepam olefin bond with hydrogen peroxide.**Ionization:****Oxidation:****Figure 4** Ionization and oxidation steps in carbanion oxidation.**Oxidation Mediated by SET to Dioxygen**

While the oxidative mechanisms outlined in sections “Autoxidation” and “Oxidation by Organic Hydroperoxides and Hydroperoxide” above can explain a large number of oxidations, certain electron-rich moieties or compounds can undergo a “direct” SET to dioxygen. Two general groups of such compounds will be briefly considered here. The first is oxidation of carbanions, which has been generally treated or discussed in terms of base-catalyzed autoxidation (1). Compounds with weakly acidic hydrogen atoms, upon treatment with base, can yield carbanions which can react efficiently with dissolved oxygen. In fact, base treatment of such compounds in oxygen saturated solutions can proceed at very high reaction rates so as to be synthetically useful and has been reviewed (24). One view of the mechanism of the new C–O bond formation is by the mechanism shown in Figure 4 (25,26). After ionization, the carbanion undergoes SET to oxygen to give a carbon radical and superoxide radical. This “caged” radical pair is viewed to undergo geminate recombination (after one of the unpaired electrons undergoes a spin-flip) to form the hydroperoxide anion product. It is important to note that antioxidants such as BHT and BHA will have no effect in slowing the oxidation when geminate

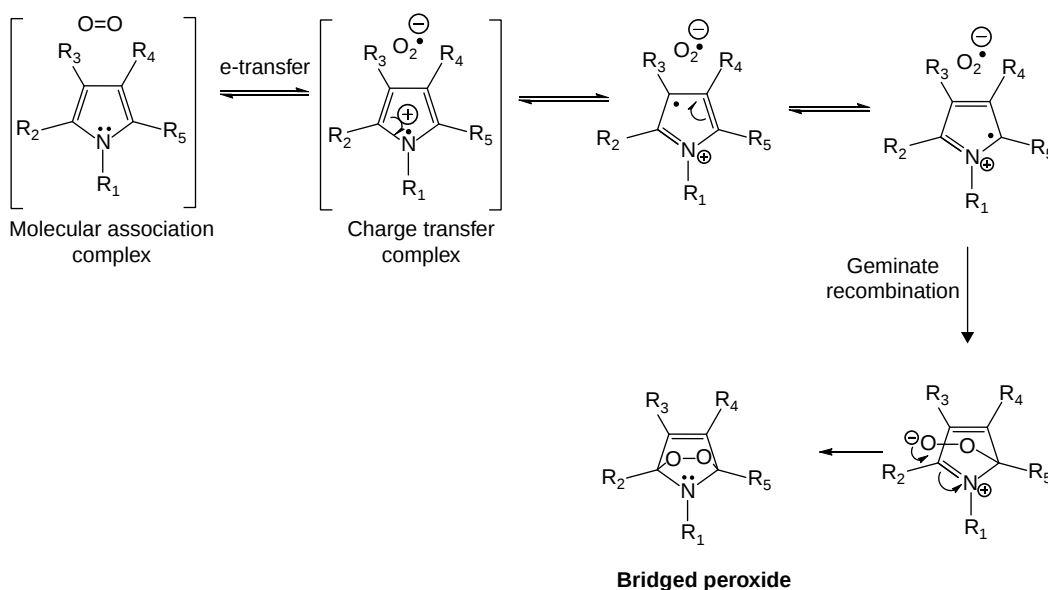


Figure 5 Direct oxidation of substituted pyrrole adapted from Ref. 29; peroxide shown is one of numerous metastable products possible.

recombination dominates (Fig. 4) rather than escape of the radicals from the solvent cage, since there are no radical chains as described in section “Autoxidation.” In a more pharmaceutically relevant context, Gu et al. (27) have reported such an oxidation mechanism for the autoxidation of ketarolac tromethamine in aqueous solution. Similarly, the oxidation of rofecoxib in higher pH solutions has been shown to proceed by this mechanism (28). Finally, a last example from this author’s experience is certain amorphous salts of phenolate-containing drug candidates may have enough carbanion character (through contributing resonance structures) to be oxidized in the solid state as in Figure 4.

The second category of compounds, in which SET to oxygen can occur, is from electron rich, but formally neutral compounds. This has been demonstrated in compounds such as pyrroles (29), α,β -unsaturated enamines (30), sterically strained cyclic olefins (31), and strained aromatic polycyclic compounds (32). The driving forces for SET are a high energy level of the highest occupied molecular orbital or a steric strain of the starting molecule. Complexation of oxygen by the electron-rich organic molecule has often been indicated as the first step of the mechanism. As such, a first-order kinetic curve in oxygen partial pressure is expected and is strong evidence of the direct oxidation mechanism, since autoxidation in Scheme 2 does not depend on oxygen pressure. Figure 5 shows the mechanism for a substituted pyrrole adapted from Beaver et al. (29). In the case of initially neutral compounds, the geminate recombination initially gives a cation and the hydroperoxide anion which may further react to give a bridged peroxide (Fig. 5). This type of pyrrole bridged peroxide oxidation product was recently implicated in the degradation profile of a pyrrole-containing pharmaceutical compound formulated in tablets and stored at 40/75% RH for 4 weeks (33). Although a mechanism was not explicitly offered, the current authors feel that a mechanism as in Figure 5 is very likely. A final example of this type of oxidation is retinoic acid, which can be oxidized by molecular oxygen without the need for radical initiators (34). The key evidence was the isolation of the product in Figure 6 that cannot be accounted for by the mechanism outlined in section “Autoxidation” but is predicted by the electron transfer mechanism in Figure 5.

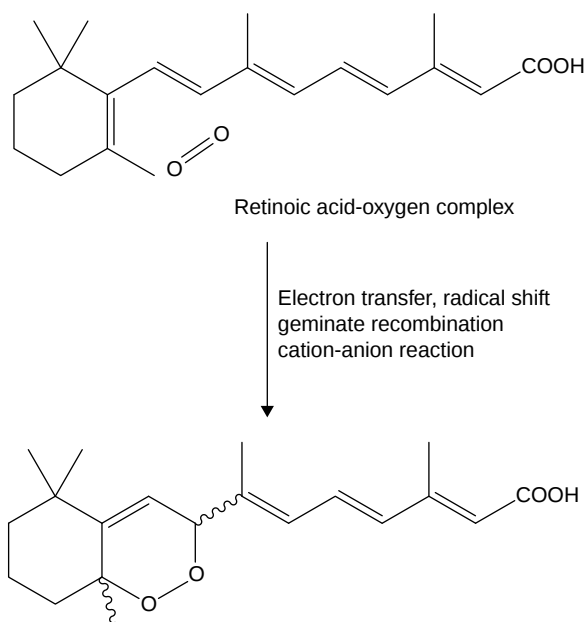


Figure 6 Retinoic acid and its cyclic peroxide supporting a direct electron transfer to oxygen mechanism.

PRACTICAL TESTS AND CONSIDERATIONS FOR OXIDATIVE SUSCEPTIBILITY TESTING

The aim of oxidative susceptibility studies is to accelerate these “natural” oxidation processes described above, so that the intrinsic selectivity of the oxidants described in sections “Autoxidation,” “Oxidation by Organic Hydroperoxides and Hydroperoxide,” and “Oxidation Mediated by SET to Dioxygen” is maintained. Here “natural” means “occurring in solid dosage forms under normal long-term storage conditions and potentially under catalysis of common (tablet) excipient impurities. Since these studies are designed to mimic normal long-term oxidative processes, they can be thought of as “predictive” oxidative tests. Three goals of such testing can be identified:

1. To predict whether the substance is particularly sensitive to oxidation or not (semi-quantitative prediction). This enables comparisons to other drug substances so that formulation options/issues may be understood very early in development.
2. To discover specific oxidative degradation mechanisms, in order to prevent the degradation (e.g., Is oxidation peroxy radical mediated or SET to molecular oxygen?).
3. To produce the oxidative impurities profile that may be formed under accelerated and long-term storage conditions. This information facilitates development of appropriate stability indicating chromatographic methods.

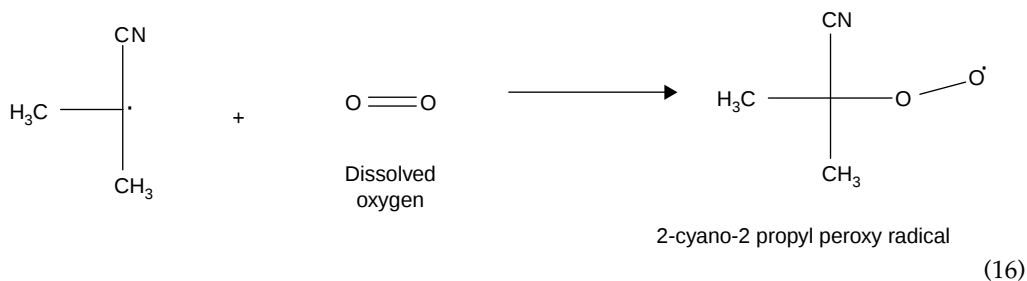
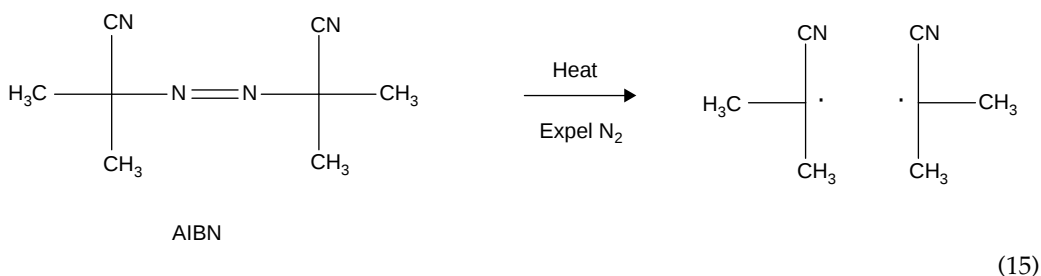
It is appropriate to mention here that other oxidative reagents and conditions may be appropriate for conducting *investigations* into oxidative degradation pathways. The purposes of such investigations include (a) the desire to make larger quantities of individual degradation products for structure elucidation or other purposes (via selectivity or faster kinetics) and (b) explorations of mechanistic aspects of the oxidative degradation pathways. Some suggested reagents and conditions for conducting oxidative investigations are shown in chapter 20, The remainder of this section will be organized similarly to section “Mechanistic Background for the Most

Common Oxidation Routes,” focusing on practical, “predictive” tests and experimental aspects for determining the susceptibility of a compound to autoxidation, to oxidation by organic hydroperoxides and hydrogen peroxide, and to explore the potential for direct SET to oxygen. A key common theme is controlling experimental conditions to selectively produce the desired oxidants in the test solutions.

Autoxidation

Use of Azo Compounds to Generate Peroxy Radicals

The autoxidation in Scheme 2 is selective due to the reactivity of peroxy radicals with low bond energy C–H bonds (or addition to olefin bonds) as described above. It is thus paramount to have a methodology which can generate exclusively peroxy radicals $\text{ROO}\cdot$ in the subject test. Azo compounds (common examples in Fig. 7) including the popular azobisisobutyronitrile (AIBN), are well-known organic reagents capable of generating peroxy radicals by thermal decomposition in solution as shown in Eqs. (15) and (16) below for AIBN:



Upon heating, azo compounds expel nitrogen to generate carbon centered radicals which rapidly oxygenate at the diffusion controlled rate in oxygen saturated solutions to form 2-cyano-2 propylperoxy radical (in the case of AIBN). In the context of Scheme 2, this peroxy radical then “replaces” $\text{R}_{\text{imp}}\text{OO}\cdot$ in Eq. (5a) and serves to initiate the oxidation of the substrate by its own peroxy radical [Scheme 2, Eqs. (6) and (7)]. Thus, when azo compounds are used in relatively low abundance with respect to an oxidizable substrate, Scheme 2 is a fair representation of the oxidation which takes place. In fact, much of the original work examining reaction rates of peroxy radicals with organic substrates was carried out in this limit. Azo compounds were typically used at only a few mole percent relative to the oxidizable substrates being studied (35,36), and substrate concentrations were high (often neat liquids). In this limit, the azo compound-derived peroxy radicals readily abstract hydrogen atoms from substrate and are converted to stable hydroperoxides.

However, the use of azo compounds by the pharmaceutical industry for the subject oxidative test has evolved to a different limit in which the azo compound “initiator” is often used in significant molar excess compared to the dilute drug substance, and substrates are much more dilute. This can have a negative impact on the selectivity of the oxidative test, as we shall

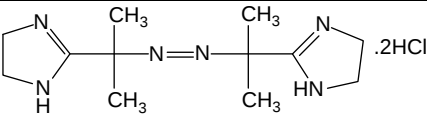
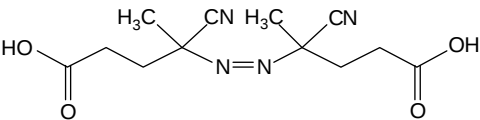
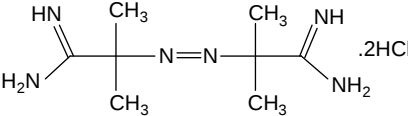
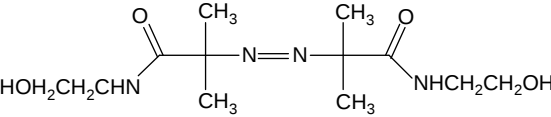
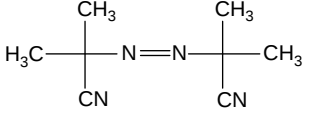
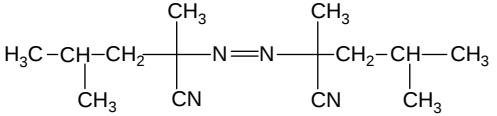
Chemical name	Structural formula	10 hour half-life	Solubility
2,2'-Azobis(<i>N,N'</i> -dimethyleneisobutyramidine) dihydrochloride		44C in water	35.2 mg/mL in water
4,4'-Azobis(4-cyano-pentanoic acid), ACVA		69C in water	1 mg/mL in water
2,2'-Azobis (2-amidino-propane)dihydrochloride, AAPH		56C in water	23.2 mg/mL in water
2,2'-Azobis[2-methyl-N-(2-hydroxyethyl) propion-amide]		86C in water	2.4 mg/mL in water
2,2'-Azobisisobutyro nitrile, AIBN, VAZO64		65C in toluene	7.5 mg/mL in methanol
2,2'-Azobis(2,4-dimethyl-valeronitrile, VAZO52, AMVN		51C in toluene	22 mg/mL in methanol

Figure 7 Common azo compounds used in oxidative stress testing shown from top (least water soluble) to bottom (most water soluble).

see shortly. At this point, a historical overview of the azo compound to substrate mole ratio used in pharmaceutical applications is warranted. The first use of azo compounds to examine autoxidation of pharmaceutical compounds in solution was Oyler in 1991 (37), Boccardi in 1992 (23), and Boccardi in 1994 (38). In these cases, AIBN was in molar excess compared to the drug substance, the AIBN/drug molar ratio being about 2:1 (60 mM AIBN/35 mM drug) and 10:1 (170 mM AIBN/15 mM drug) for Boccardi and Oyler, respectively. These studies, in particular Boccardi's detailed work on tetrazepam (5,38), established the potential of azo compound "initiation" in solution to mimic autoxidation in solid dosage forms and started a more general usage of azo compounds for examining the oxidation potential of drug substances. By 2003, Alsante et al. (39) surveyed the pharmaceutical industry in regard to forced stress testing practices and found that azo compound initiators were commonly used with AIBN in water-acetonitrile-based solvent systems being typical. Currently, drug substances are being examined more and more early in development, when only mg quantities may be available. As a result, common drug concentrations for the subject test in a current pharmaceutical context have significantly diminished, while the azo "initiator" to drug molar ratio has remained near 10 to 1 (typical concentrations currently might be about ~5 mM azo compound and ~0.5–1 mM drug substance).

This sets the stage for potential selectivity problems in the current pharmaceutical context of the azo compound experiment. Let us assume based on bond energies that peroxy radical reaction rates with common a solvent such as acetonitrile (or methanol) are negligible (15).

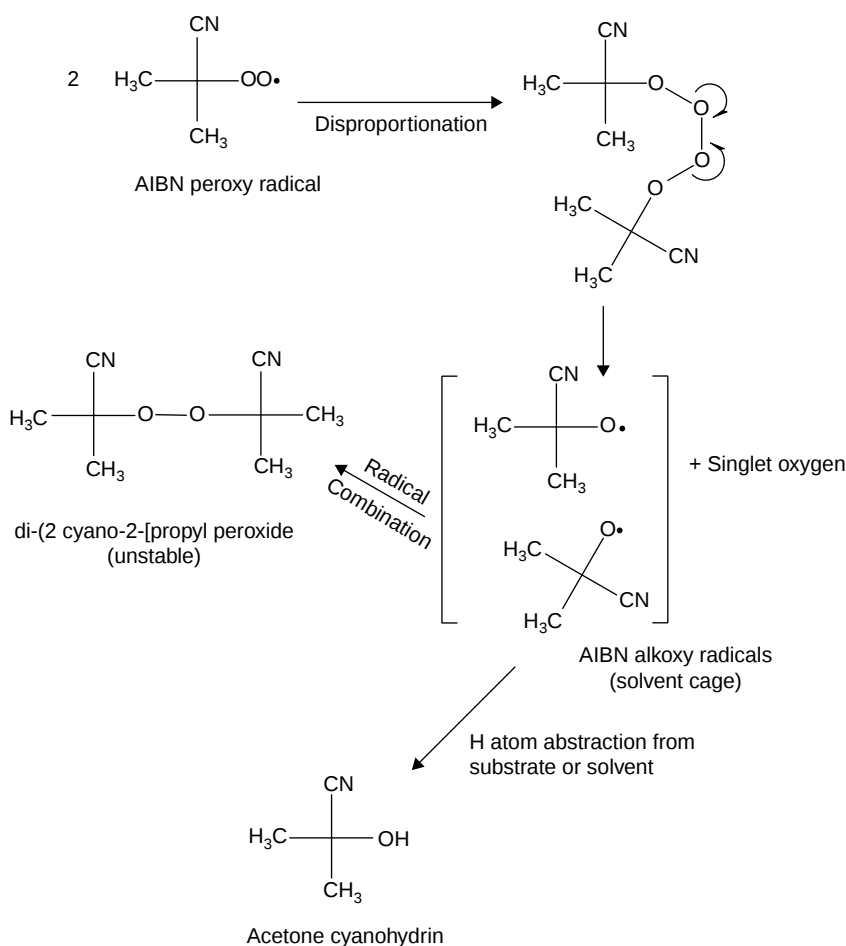


Figure 8 Disproportionation reaction of AIBN peroxy radical in the absence of any oxidizable substrate or solvent, yielding 2-cyano-2 propoxy radical.

Then, at large azo compound to substrate molar ratios, with dilute substrates that are potentially not reactive or only moderately reactive toward peroxy radical, some disproportionation of the azo peroxy radicals can be expected since rate constants for peroxy radical disproportionation are large compared to any C–H bond abstraction rates [on the order of $2k_t \sim 10^3\text{--}10^7 \text{ M}^{-1} \text{ s}^{-1}$ (10,18)]. This disproportionation reaction is shown specifically for AIBN in Figure 8. The selectivity issue is that all azo compound peroxy radicals are *tertiary* peroxy radicals, and as such, disproportionation of these peroxy radicals must generate alkoxy radicals, $\text{RO}\cdot$. The bond energies of $\text{RO}\text{--H}$ species are near 105 kcal/mole (15) as compared to that of 89 kcal/mole for $\text{ROO}\text{--H}$ bonds as discussed previously. Thus, alkoxy radicals are not selective in that they will react with much stronger C–H bonds than peroxy radicals. This would signal “incorrect” (i.e., nonpredictive) oxidative degradation profiles. Further, even if the alkoxy radicals in Figure 8 reacted with the “appropriate” C–H bond (i.e., a C–H bond that peroxy radicals can also react with) that reaction rate will typically be $10^4\text{--}10^6$ -fold larger than H-atom abstractions by peroxy radicals (36). For example, the ratio of *tert*-butoxy radical to *tert*-butyl- peroxy radical H-atom abstraction rates for cumene, tetralin, tetrahydrofuran, and toluene are $\sim 10^6$, $\sim 4 \times 10^5$, $\sim 1.5 \times 10^5$, and $\sim 4 \times 10^5$, respectively (36). Thus, in addition to the selectivity issue, relatively low levels of

“strong” alkoxy radicals (2-cyano-2 propoxy radicals in Fig. 8) could also “signal” significant peroxy radical reactivity of the dilute drug substance when in fact, there was very little reactivity. Either case is a significant issue for the subject test.

Choice of Solvent Composition to Minimize Alkoxy Radical Activity

Several examples of solvent effects for azo compound oxidation related to Figure 8 have in fact been recently reported in the pharmaceutical context and will be briefly described here. Using 5 mM AIBN or ACVA in 100% acetonitrile with 0.5 mM cumene (0.1 mg/ml) as a substrate (a model for a dilute drug substance), Nelson et al. (40) found that the total oxidation products of cumene decreased 5-fold (for ACVA) and ~10-fold (for AIBN) upon addition of only ~2–5% by volume methanol to the acetonitrile test solution. The authors argued that the actual “oxidant” in the absence of methanol solvent was in fact the AIBN-related alkoxy radical in Figure 8, and that the addition of even small amounts of methanol served to “quench” the 2-cyano-2 propoxy radical (by rapid donation of a H atom by the methanol). The expected methanol oxidation products were detected (i.e., formic acid and formaldehyde). As further supporting evidence, the authors noted that in the case of cumene oxidation, the cumene alkoxy radical can serve as a type of “internal clock” that is sensitive to the effective H atom donation ability of the solvent. The tertiary cumene alkoxy radical (formed by disproportionation) undergoes an internal β -scission rearrangement to form acetophenone. This rapid internal rearrangement competes with H-atom abstraction from solvent to yield 2-phenyl-2-propanol. The acetophenone/2-phenyl-2-propanol product ratio was found to be steadily reduced by 2-fold by addition of only 1–3 % methanol (vol%) to the acetonitrile. This was interpreted as ca. 50-fold higher H-atom donation rate by methanol to the cumene alkoxy radical (as compared to acetonitrile). This supported the authors’ proposed rapid H-atom donation rate of methanol (compared to acetonitrile) to the 2-cyano-2 propoxy radical.

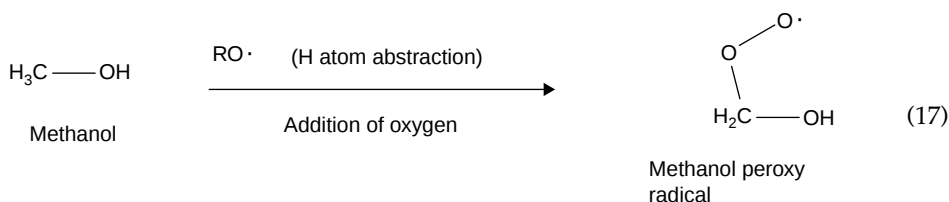
Watkins et al. (41) have also reported similar results in which the addition of low levels of methanol to acetonitrile/water AIBN and ACVA initiated oxidation experiments eliminated significant (3–10% levels) oxidative degradates which would have otherwise been presumed to be “peroxy radical” mediated. These authors isolated the degradation products and showed by NMR structural elucidation that, in fact, the degradation was from the addition of the 2-cyano-2 propoxy radical to an aromatic ring system. This result unequivocally demonstrates the alkoxy radical activity shown in Figure 8 in acetonitrile-water cosolvent systems under current pharmaceutical azo compound stress testing conditions (i.e., in the presence of acetonitrile without the presence of methanol). Methanol H-atom donation to the alkoxy radical was again rationalized as the mechanism by which methanol removed this unwanted reactivity. Once the *alkoxy* radical activity was quenched, there was no observed degradation of the drug from the remaining *peroxy* radicals generated by either AIBN or ACVA (41).

Given that this azo-derived alkoxy radical activity may be somewhat surprising to practitioners in the field, and that it can significantly undermine the subject oxidative test result conclusions for substrates unreactive toward peroxy radicals, several other considerations regarding the reaction shown in Figure 8 should be detailed. High yields of acetone cyanohydrin, derived from H-atom abstraction of the 2-cyano-2 propoxy radical as shown in Figure 8, have been shown for the AIBN-initiated oxidation of neat benzene (85% yield) and neat xylene (20% yield) in oxygen-saturated solutions using 0.6 M AIBN with a reaction temperature of 50°C (42). These data clearly show efficient disproportionation, as shown in Figure 8, even in the presence of *molar* concentrations of marginally reactive substrates (benzene and xylene).

Another point to consider in understanding the “reality” of the reaction in Figure 8 is the relative amount of AIBN peroxy radicals formed, compared to the dilute drug substance oxidized. Using the known Arrhenius parameters for AIBN decomposition, and a mean oxygenation efficiency of 50%, Boccardi (5) estimated that after 2 days at 40°C, the yield of the 2-cyano-2 propoxy radical will be 5% relative to the starting AIBN concentration. Taking this 5% conversion value and the typical 5 mM AIBN concentrations used, over a 2 days’ test period 0.25 mM 2-cyano-2 propoxy radical will be generated. This can be compared to the amount of drug

oxidized in a “typical” case, for example, assume 5% degradation of the initial 0.5 mM drug substance, and thus, 0.025 mM drug is degraded. Over the 2 days’ test period there is 10X more 2 cyano 2 propyl peroxy radical generated than drug oxidized, and the disproportionation in Figure 8 is inevitable if the solvent is also unreactive with peroxy radical.

It is possible that some of the azo compounds in Figure 7 may have different effective yields of their own alkoxy radicals, or that the alkoxy radical yields have some solvent dependence. However, a general solution to the azo compound disproportionation problem is to recommend that at least 10% methanol should be added to acetonitrile–water solvent systems to quench any potential azo compound alkoxy radical activity. Alternatively, methanol–water and or ethanol–water cosolvent systems (without any acetonitrile) will offer the same alkoxy radical quenching advantages and should not otherwise significantly alter overall peroxy radical-mediated oxidative yields or degradation profiles (38). However, if hydrolytically sensitive intermediates (such as epoxides) are generated, then use of high methanol- or ethanol-based solvents may give different degradate profiles due to solvolysis. Note that the H-atom donation by methanol to an alkoxy radical produces a methanol radical that will oxygenate to form a methanol peroxy radical as shown in Eq. (17), which should have the appropriate selective reactivity for the subject test. For completeness, it should be pointed out that this methanol oxidation will lead to formaldehyde and formic acid (at a few μM) in the sample (41):



Oxygenation Requirements

As described in section “Autoxidation”, expressions for the rate of autoxidation in solution are proportional to the product of the substrate concentration and the rate constant of the peroxy radical hydrogen atom abstraction, which is relatively slow. Given the oxygen solubility in liquids in equilibrium with ambient oxygen and the rapid rate of Eq. (6) in Scheme 2, there is typically no oxidation rate dependence on the dissolved oxygen concentration (18,19). Thus, the typical experimental conditions for the subject autoxidation test in regard to oxygenation has been to use unstirred solutions, in capped flasks with roughly an equivalent solution volume of ambient atmosphere over the solution. Given a range of potential initiator concentrations which could be used and widely varying substrate reactivities, it is reasonable to consider if enough dissolved oxygen remains in solution during an autoxidation test as just described. Some investigators have used enriched or pressurized oxygen atmospheres to ensure a maximal degradation rate in azo-initiated oxidation experiments. Many researchers feel some reticence in enriching oxygen levels in combination with the potentially explosive azonitriles. Fortunately, some measurements have recently been made which are informative. Nelson et al. (43) measured oxygen remaining in the headspace over equal volumes of AIBN and ACVA in solution at 1, 5, 25, and 50 mM in the absence of substrate at 40°C. Figure 9 shows the AIBN data for oxygen levels remaining in the headspace over 7 days; both with and without stirring of the solutions. After 2 days, there is little depletion observed for the 1 and 5 mM AIBN levels both with and without stirring; even the 25 mM AIBN cases are only ca. 20% depleted, and only marginal differences between stirred and unstirred. The presence of substrate will increase the oxygen consumption and needs to be considered briefly. Assuming drug substance at 0.5 mg/mL or 1 mM, and again using the estimated 5% AIBN yield over 2 days (5), we can estimate that the oxygen consumption in Figure 9 corresponds to 0.25 mM AIBN peroxy radical formed. This corresponds to 25% of the drug present. Thus, even if *all* the drug were consumed by subsequent

propagation steps, the total oxygen consumed would be 4X greater- or about similar to the 25 mM AIBN data in Figure 9 (around 20% depleted). Thus, the data support the current general practice of working with ca. 5 mM AIBN levels in unstirred solutions. The AVCA data (43) show about 2-fold more oxygen consumption, and similarly supports 5 mM initiator concentrations (and low mM drug concentrations) in unstirred flasks.

Figure 9 does highlight that use of significantly higher initiator and drug concentrations (50 mM) will likely lead to substantial depletion of oxygen in the bulk solution. While it is by no means clear that this would adversely affect the general results of the subject test, it is important nonetheless to be aware of the resulting nonlinear kinetics and the potential for alterations in degradation profiles.

Solution pH and Temperature

Generally, control of the solution pH during azo compound-initiated oxidation has not been highlighted in the literature. However, given the abundance of amine groups in pharmaceutical compounds, it is plausible that C–H bonds adjacent to amine groups could have a susceptibility to H-atom abstraction by peroxy radicals which is dependent on the protonation state of the nitrogen atom lone pair of electrons. Figure 10 shows this effect quite elegantly for a peroxy radical-mediated oxidation of a pyrrolidine ring to an aromatized pyrrole ring. There

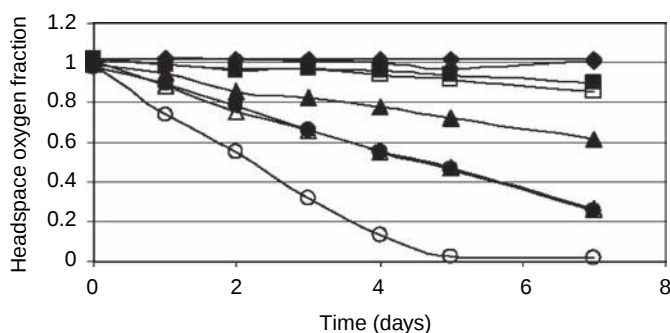


Figure 9 Headspace oxygen concentration as a function of time for 50% acetonitrile solutions containing (◆) 1 mM AIBN, (♦) 1 mM AIBN with agitation, (■) 5 mM AIBN, (□) 5 mM AIBN with agitation, (▲) 25 mM AIBN, (△) 25 mM AIBN with agitation, (●) 50 mM AIBN, and (○) 50 mM AIBN with agitation. Solutions were stored at 40°C and either stirred or left static in between measurements. Oxygen levels are normalized to the results measured each day for a 50% acetonitrile control sample.

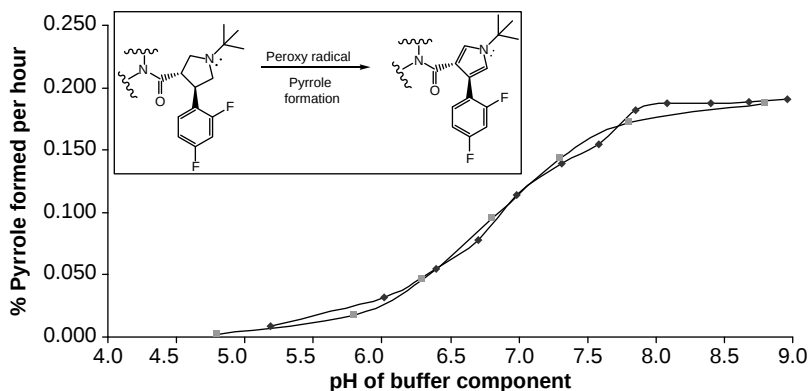


Figure 10 pH dependence of the AIBN oxidation of a drug molecule to form a pyrrole moiety. Gray squares (calculated pH curve, described in the text), and black diamonds are experimental values.

is a net mass loss of 4amu from the parent compound; the conversion can be envisioned as resulting from two reactions with peroxy radical and two subsequent eliminations. Figure 10 shows the relative rate of the pyrrole formation (as a % initial drug peak area) as a function of the pH of the 50% aqueous (20mM phosphate buffer) portion of the solvent; the other 50% being methanol. The black data points and solid line are the data; the gray data points and curve are to guide the eye and are a simple equilibrium pH calculation of normalized concentration of the deprotonated form of an amine group with a pK_a of 6.8 in the mixed solvent system. The data clearly show that the overall pyrrole oxidation rate is controlled by the protonation state of the pyrrolidine ring nitrogen atom. The pyrrole oxidation rate increases 20-fold from an apparent pH 5.0 to 8.0. Figure 10 highlights that if this compound were available as an HCl salt versus a free base, and tested at 0.5mM in an unbuffered acetonitrile/methanol cosolvent system, two considerably different pyrrole oxidation yields would likely be obtained. These data warrant considering pH control of azonitrile-initiated oxidations if the substrate has amine (or other) moieties with pK 's in the pH range of ~ 4 to ~ 9 in the mixed solvent system.

The final experimental variable to be considered is the temperature of the oxidation experiment. The temperature is a balance between the need for the thermal decomposition of the initiator (Eq. (15)) and trying to minimize thermal degradation of hydroperoxides or peroxides as shown in Eqs. (18) and (19):



Generation of these strong alkoxy and hydroxyl radicals would degrade the selectivity of the subject test as described previously in detail for $\text{RO}^\bullet$ radicals. Hydroxyl radical is similar in that it is a much stronger radical than peroxy radical. Given the estimation described here of 5% yield of AIBN peroxy radicals over 2 days at 40°C, and the typical drug concentrations used, 40°C is recommended as the base case temperature to carry out the subject test. Higher temperatures could be used, but the contribution of total oxidative products by oxidation via Eqs. (18) and (19) would be increasingly difficult to determine. This could mask overall reactivity of the substrate with peroxy radical, which is one primary outcome of the experiment. Higher temperatures also reduce dissolved oxygen concentrations. Table 1 summarizes the azo compound oxidative test conditions recommended here.

Table 1 Recommended Conditions for the Oxidative Susceptibility Tests

Test	Desired Oxidation Mechanism	Temperature	Concentrations of Oxidant	Concentrations of Substrate	Solvent Composition	pH Control?	Duration
Hydrogen peroxide	2 electron oxidation	≤RT	≤0.3% by volume	0.1–1 mM	water/ACN with methanol	Yes if amines	≤24 hours
AIBN	Peroxy radical oxidation	40°C	~5 mM	0.1–1 mM	≥10% methanol, ACN, water	Yes if amines	≤48 hours
Transition metals	Electron transfer	40°C	~1 mM	0.1–1 mM	ACN/water (low methanol)	Neutral pH	≤72 hours

Alternative Peroxy Radical-Based Oxidative Stressing System

The azo compounds in Figure 7 are toxic and can explode under certain conditions. For these reasons, some laboratories have difficulty obtaining azo compounds. There has been another methodology reported for generating a peroxy radical-based oxidative stressing system without the use of azo compounds (44). This oxidative system leverages Scheme 1. Tween 80 is used at 10% by weight in aqueous solution to provide high levels of ROOH and Fe(III) is added at 10 mM. Although peroxy radicals are formed along with alkoxy radicals (Scheme 1), the 100 mg/mL levels of oxidizable Tween 80 reacts with those alkoxy radicals prior to encountering sub-millimolar concentration substrates thus preserving the peroxy radical activity. General oxidizability rankings with this system appear consistent with AIBN-initiated systems (44). One significant disadvantage of the Tween 80/Fe(III)-based system is that Tween 80 is not amenable to subsequent LC-MS analysis of the oxidative degradates formed.

Oxidation by Organic Hydroperoxides or Hydrogen Peroxide

Section "Oxidation by Organic Hydroperoxides and Hydroperoxide" highlighted the potential reactions of hydrogen peroxide or organic hydroperoxides ROOH with drug molecules. The intact hydroperoxide may act as either an electrophile or a nucleophile. Experimental conditions must be optimized to ensure that this two electron or paired electron reactivity is the only significant oxidative reaction possible during the test measurement. Thus, critical experimental parameters are the test temperature, the solvent composition, and the concentration of hydrogen peroxide. These will be considered in sections "Hydrogen Peroxide Level and Temperature" and "Water Cosolvent System". In section "pH of Cosolvent System", we will discuss the effects of the test solution pH in the case where drug substance amine group reactivity is being explored.

Hydrogen Peroxide Level and Temperature

The two electron reactions being probed with the hydrogen peroxide test are relatively rapid at room temperature, and thus there is little need to elevate reaction temperature beyond ambient. Increased reaction temperatures come with higher risk associated with creation of additional undesired hydroxyl radical oxidants as shown in Eq. (20). The peroxide bond in hydrogen peroxide has a bond energy of about 213 kJ/mol (50 kcal/mol) (45). Homolytic decomposition of hydrogen peroxide will increase in rate as the temperature is raised:



Hydroxyl radicals are strong, nonselective oxidants as described above, and can rapidly react with drug substances in solution and confound the normal peroxide (i.e., paired electron) reactivity being examined in the subject test.

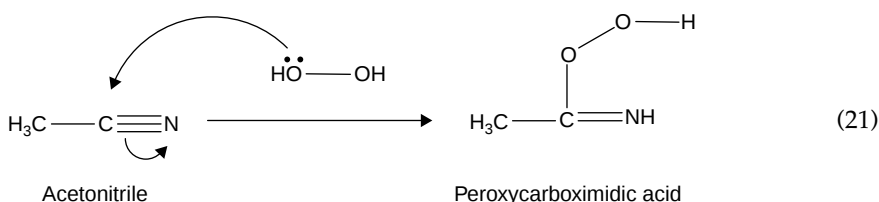
Hydrogen peroxide concentrations do not need to be more than 0.3% (which corresponds to ~90 mM) and can often be 10-fold lower (~9 mM) as the paired electron reaction rates are generally much faster than peroxy radical reactions. Drug concentrations are convenient to use at submillimolar concentrations rationalized in section "Autooxidation". A stress period of 24 hours should be adequate time to show a reaction with hydrogen peroxide (or, as important, to show the lack of a reaction).

Water Cosolvent System

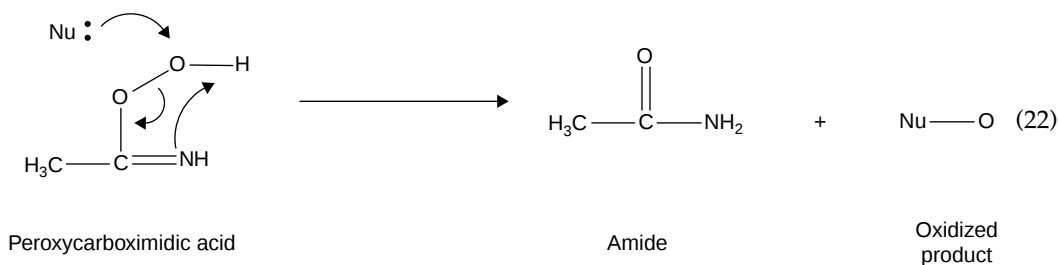
It is very important that a cosolvent be used in addition to water. Methanol and acetonitrile will serve to "quench" any low-level hydroxyl radical activity produced by Eq. (20) (even at room temperature) by donation of a hydrogen atom to the hydroxyl radical. In this regard, it has been this author's practical experience that methanol appears to be a better H-atom donor to hydroxyl

radical than acetonitrile, similar to the conclusion reached by Nelson et al. (41) regarding methanol being a better H-atom donor to the 2-cyano-2 propoxy radical compared to acetonitrile. Thus at least 20% methanol is recommended as a cosolvent (remaining being water, acetonitrile, or methanol). It should be recognized that cosolvent quenching of hydroxyl radicals thus leads to low levels of solvent peroxy radicals (as shown in Eq. (17) for H-atom donation of methanol). Therefore, minor peroxy radical degradate peaks may also form over the 24-hour hydrogen peroxide test duration. Any hydroperoxide paired electron reactivity should be much larger in comparison.

There is, however, a clear preference for methanol as a cosolvent if a higher pH range of the hydrogen peroxide reaction with the drug substance needs to be explored (as described in the next section). At higher pH in a water–acetonitrile cosolvent system, acetonitrile has the liability of being able to react with hydrogen peroxide to form peroxycarboximide acid (46,47) as shown in Eq. (21):



Peroxycarboximide acid is an unstable oxidizing species; it is even more reactive than hydrogen peroxide as it can undergo reaction with even weaker nucleophiles (Nu in Eq. (22)) due to the more favorable leaving group compared to hydrogen peroxide:



Since a drug substance will not encounter peroxycarboximide acid in a pharmaceutical dosage form, any oxidized product formed as in Eq. (22) is an unnatural and undesired oxidation event for this test. Methanol/water or ethanol/water are more appropriate and common solvent systems which avoid this problem and provide excellent solubility for most drug substances.

pH of Cosolvent System

The reaction of amines with hydrogen peroxide shown in Eqs. (11) and (12) will slow dramatically when the nitrogen lone pair of electrons is protonated. Thus, some attention should be given to the cosolvent apparent pH during the hydrogen peroxide test. Figure 11 shows the typical trends one can expect for a tertiary amine. The cosolvent system is 50% methanol, 50% phosphate buffer adjusted to the pH values shown on the *x* axis. The hydrogen peroxide concentration is 0.3%, the drug is present at 0.1 mg/mL and the stress temperature is ambient. The *y* axis gives the *N*-oxide formation rate observed (determined from the slope to a

linear fit of four data points over the first 6 hours) in % total drug present converted to the *N*-oxide (per hour). The black data points show the actual data, while the gray data points are to guide the eye and derive from a simple calculation of the protonated/deprotonated amine nitrogen ratio based on an apparent pK_a of 7.0 in the mixed solvent system. Thus, the protonation state of the amine nitrogen is controlling the reaction rate as expected. The simplest recommendation would be to carry out the hydrogen peroxide test with the pH controlled near the pK_a of the amine in the cosolvent system if amine groups are present. In Figure 11, this gives ~10% formation of tertiary *N*-oxides with overnight stressing. Note that if the reaction had been inadvertently carried out at an apparent pH near 4, the entire *N*-oxide formation might have been missed as a potential oxidation route. Table 1 summarizes the recommended test conditions.

Oxidation Mediated by SET to Dioxygen

In studying the reactivity of a new drug substance, it is obviously interesting to ascertain whether it can undergo SET to molecular oxygen at an appreciable rate, but this is often not a simple task. An obvious step is to start by monitoring an oxygen-saturated solution of the test compound with no azonitrile initiator or hydrogen peroxide added. However, bearing in mind the trace levels of initiators acting in Scheme 1 and their efficacy at very low concentrations, it is practically impossible to exclude them totally. Nevertheless, the first clue of an oxidation mediated by direct electron transfer to oxygen is oxidative degradation in experiments performed with particular care using very pure reagents and very clean apparatus. Often there appears to be no significant induction period to the oxidation. The most convincing evidence, as discussed in section "Mechanistic Background for the Most Common Oxidation Routes", is that the oxidation will typically show a first-order dependence on the dissolved oxygen concentration even at ambient (saturated) dissolved oxygen levels. However, there are some experimental approaches to be considered in the context of gauging the susceptibility of a compound to undergo SET to oxygen. In the current context, we will consider oxidation of the substrate by Fe(III) and Cu(II) transition metal complexes as well as application of electrochemical methods, and will discuss the former first.

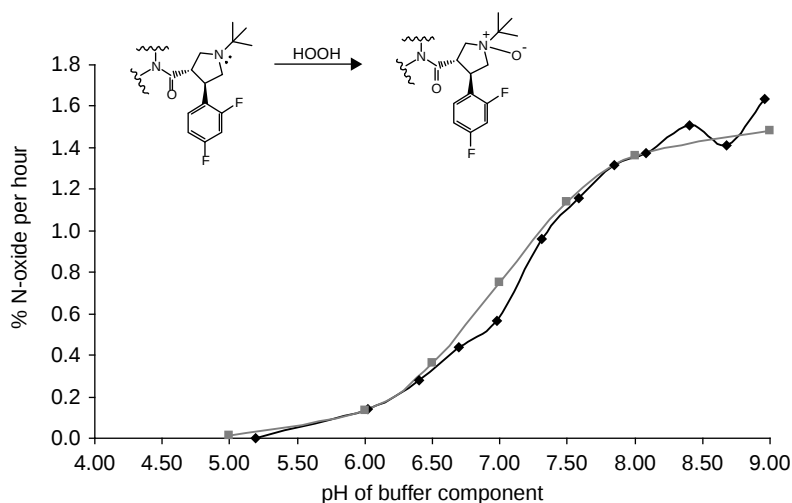


Figure 11 Formation of tertiary *N*-oxide as a function of pH of the aqueous phase of the methanol-phosphate buffer cosolvent system. Gray squares are calculated pH curve, and black diamonds are experimental data.

Use of Fe(III) and Cu(II) Transition Metal Ion Complexes

Transition metal complexes may catalyze oxidation in a number of ways. A brief discussion is worthwhile to frame any relevance of such tests to the potential for SET to dioxygen. Four very general modes of metal ion catalysis of oxidations can be considered (for a review, see Ref. 10):

1. metal ion oxidation–reduction reactions with hydroperoxides as shown in Scheme 1;
2. metal ion complex activation of molecular oxygen;
3. direct reaction of metal with substrate- “outer sphere” or electron transfer; and
4. direct reaction of metal with substrate- “inner sphere” or ligand transfer

In the context of possibly probing a drug candidate’s propensity for SET to dioxygen then, we are primarily interested in mechanism (*iii*) above, in which the coordination sphere of the metal ion remains intact. However, it is not straightforward to distinguish this from mechanism (*iv*) which involves a coordination of the metal ion with the substrate followed by electron transfer (10). The “ease” of the electron transfer in both mechanisms (*iii*) and (*iv*) will generally be related to the ionization potentials of the substrates. In either case, in our view transition metal complexes of iron(III) and copper(II) are appropriately discriminating agents for this type of test as their redox potentials in aqueous solution are not excessively positive, being near 0.77 and 0.15 V (versus NHE), respectively. For example, Harmon et. al (9) found in control experiments that 10 mM Fe(III) chloride in water/acetonitrile mixtures did not give significant oxidation of any of the 18 compounds being studied in that work. In contrast, Boccardi (23) found that 1.5 mM Fe(III) chloride and 1.5 mM Cu(II) sulfate (in acetonitrile) oxidized 40–50% of the initial tetrazepam present. These data indicate the selectivity of the subject iron(III)- and Cu(II)-based test.

Iron(III) and Cu(II) do not typically participate in mechanism (*ii*) as long as no source of reducing equivalents are present and mechanism (*i*) above is obviated by the use of clean simple solutions of the drug being studied. Thus oxidation observed during this metal ion test can generally be interpreted as due to electron transfer mechanisms (*iii*) or (*iv*) above. While neither process may predict SET to oxygen, this test does provide a simple means to get a measure of the electron transfer potential of the drug substance. Typical solvent test conditions would be acetonitrile/water solvent mixtures; however it is recommended that a small amount of methanol be added to reduce any alkoxy radical activity that might be generated. Reaction temperature is recommended $\leq 40^{\circ}\text{C}$, and metal ion complex concentrations at ca. 10–100 mol% relative to the drug substance. Drug substance concentrations might range from 0.1 to 1 mM depending on drug substance availability, but the concentration is not critical. Any number of salts and complexes of iron(III) and copper(II) can be used (5). Table 1 summarizes the test conditions.

Cyclic Voltammetry: Electron Transfer from Substrate to an Electrode

Cyclic voltammetry (CV) has been applied to the determination of the potentials at which drug substances in solution can be reversibly oxidized at an electrode surface (7,8). It is for this reason we chose to discuss the CV methodology in the context of methods which might shed light on substrates prone to electron transfer to dioxygen. However, the main focus of the CV work has been to examine the potential of the methodology as a general oxidative screen; similar to azo compound initiated oxidation discussed here.

For example, Lombardo and Campos (7) describe, in parallel with an HT protocol using a radical initiator (ACVA) at 60°C , a HPLC-electrochemical method using an array of 12 electrodes at potentials between -0.2 and 1.2 V (with reference to a Pd electrode). Compounds were examined and were ranked in six classes in decreasing order of oxidation sensitivity based on the observed oxidation potential. These results were compared to the % compound remaining in the ACVA initiated oxidation. While many compounds were ranked as stable by both techniques, there was an “orthogonal” aspect to data for some compounds; where for example, compounds that ranked as the most unstable by CV oxidation potential ranged from 0% to 83%

consumed in the AVCA initiated oxidation test (7). Gamache and co-workers (8) showed data obtained by using the same CV apparatus of Lombardo and Campos, on 22 known drug substances and antioxidants. The authors ranked substances as unstable or stable based on oxidation potential.

In our opinion, the main drawbacks of the application of CV electrochemical methods as a *general* oxidative screening method is the fact that anodic oxidation is an electron-transfer reaction which is not that common an oxidative pathway in solid dosage forms as discussed above. A compound could in principle be resistant to oxidation by electron transfer to dioxygen, but very prone to reaction with peroxy radical (and vice versa). In fact, it is our naïve hope that this is the nature of some aspects of the “orthogonality” noted by Lombardo and Campos. However, at this time that correlation has not been demonstrated, but efforts spent in development of CV methods focused on predicting/clarifying/correlating the potential for direct electron transfer to dioxygen would be of significant value. In this sense, our view is similar to some perspectives of Waterman and co-workers (3), who also described the use of CV to study the oxidation sensitivity of known drugs and concluded that CV is more appropriate for detailed mechanistic studies than for fast general oxidative screening.

SUMMARY AND GENERAL STRATEGY OF OXIDATIVE SUSCEPTIBILITY TESTING

Section “Practical Tests and Considerations for Oxidative Susceptibility Testing” began by highlighting primary goals of oxidative susceptibility testing- to understand a compound’s liability to oxidation early on, so as to inform formulation efforts and optimization strategies from the start. In order for this approach to work, the solution-based oxidative stress must generate only the oxidizing agents found in solid dosage forms. In that regard, peroxy radicals and organic hydroperoxides have been put forth here as the most common oxidizing agents. The careful choice of experimental conditions described here (summarized in Table 1) can reduce the effects of undesirable oxidants generated in the AIBN and the hydrogen peroxide tests, and thus provide the clearest view of the true oxidation potential.

In this context, it is useful to consider the “interpretation” of the levels of degradation one might encounter in these oxidative tests. If Table 1 is followed as recommended, it is this author’s experience that for the AIBN test in methanol containing solvents, a good number of drug substances will degrade very little if alkoxy radical activity has been eliminated. It is not uncommon to see only 0–2% drug lost during an AIBN test (41–43) and as such these compounds would be classed as oxidatively stable toward peroxy radical. Similarly many molecules (except tertiary amine containing drugs) also give 0–2% loss in the hydrogen peroxide test carried out as in Table 1. Molecules giving these types of low percentage assay loss in the AIBN and hydrogen peroxide test results can be expected not to have any issues with oxidation in solid dosage forms regardless of formulation strategy in this author’s experience. This, in fact, is the one of the best “predictions” that oxidative susceptibility testing can provide-the *lack* of sensitivity. On the other hand, a result of greater than 20% drug loss in the AIBN test signals the compound is susceptible to peroxy radical oxidation and would require careful selection of excipients and, potentially, the use of antioxidants. The hydrogen peroxide test delivers high concentrations of oxidant and should show large % conversions (5–100%) of the drug if such a two electron ionic reactivity is present. If found, it predicts some sensitivity to peroxide containing or peroxide generating excipients; but fortunately, the reaction of drug molecule with a ROOH group in this way is stoichiometric and the drug is often in large excess compared to the trace ROOH levels.

Transition metal ions such as iron(III) and copper(II) may allow detection of substances with a low redox potential. This may be useful in the context of understanding the potential for oxidation by electron transfer to oxygen. If the test compound is sensitive to a number of different oxidants or catalysts, such as AIBN, hydrogen peroxide and iron(III), it is advisable to consider the substance as potentially very sensitive to oxidation. In this case, it is wise to consider preformulation efforts investigating the use of antioxidants or special protecting conditions.

A second tier of tests should be designed for compounds already detected as sensitive. The goals of the second tier are the isolation of impurities and a more detailed investigation of the degradation mechanism. It is not possible to propose general protocols, as in this case the chemistry of the substance must be fully considered. In this phase, we can study, by comparison with known examples and data, solvent effect, pH effect, detection of hydroperoxides, or the use of singlet oxygen or oxidants that are more selective for the structural class of interest. Once the degradation profile is ascertained, more in-depth studies can be recommended to further guide preformulation activities.

FUTURE DIRECTIONS

Oxidation problems in solid dosage forms are most often caused by peroxy radical and as such, the AIBN test (or other similar compounds in Fig. 7) is likely the single most important test in determining the likelihood of potential oxidative issues in a solid dosage form. Table 1 now provides a means to reduce “false positives” in this test, in particular by careful selection of solvent composition. This improvement should allow for better semi-quantitative correlations to be developed between AIBN test “% claim lost” values and formulation routes needed (if any) to stabilize the drug substance. Published examples of data sets comparing long-term (oxidative) stability performance in pharmaceutical dosage forms and AIBN test results would be beneficial in this regard.

Another useful area of endeavor would be to document a methodology which would allow a researcher to determine if their azonitrile system was “working properly.” That is, is the azonitrile compound liberating the right amount of carbon centered radicals? Is enough oxygen reacting with those radicals to produce the appropriate amount of azonitrile-derived peroxy radical? A possible solution to this problem is to monitor the quantity of hydroperoxides (48) being generated by the system. BHA might be used in this regard as a fast H-atom donor to ensure the peroxy radicals formed are stabilized/trapped as the ROOH. Preliminary work in this author's laboratory suggests this approach may be possible. Further work is needed and other approaches could be effective.

Two final areas of investigation will be mentioned in closing. One last important area of research would be to better understand azonitrile-derived peroxy radical reactions in the limit of dilute azo compound and no substrates, other than water and acetonitrile and methanol cosolvents. This would lead to a “proof” of the mechanism by which methanol quenches the 2-cyano-2 propoxy radical activity (in the case of AIBN). Further work is also needed in terms of simple tests which help predict the potential of SET to dioxygen. Further investigations of possible correlations or “orthogonality” between CV data and azonitrile oxidation data may exist, and a unified approach to both data sets might provide a better insight into predicting SET to dioxygen.

These questions and topics will hopefully stimulate further thought and lead to additional work, approaches and insights into oxidative susceptibility testing.

ACKNOWLEDGMENT

The authors would like to acknowledge Steve Baertschi for numerous discussions and suggestions, and Francis Flanagan for generating the pH-dependent oxidation data shown in Figures 10 and 11.

REFERENCES

1. Johnson DM, Gu LC. Autoxidation and antioxidants. In: Swarbrick J, Boylan JC, eds. *Encyclopedia of Pharmaceutical Technology*. New York: Wiley, 1988; 1: 415–50.
2. Hovorka SW, Schöneich C. Oxidative degradation of pharmaceuticals: theory, mechanism and inhibition. *J Pharm Sci* 2001; 90: 253–69.
3. Waterman KC, Adami RC, Alsante KM, Hong J et al. Stabilization of pharmaceuticals to oxidative degradation. *Pharm Dev Tech* 2002; 7(1): 1–32.

4. Hartauer KJ, Arbuthnot GN, Baertschi SW et al. Influence of peroxide impurities in povidone and crospovidone on the stability of raloxifene hydrochloride in tablets. *Pharm Dev Tech* 2000; 5: 303–10.
5. Boccardi G. Oxidative susceptibility testing. In Baertschi SW, ed. *Pharmaceutical Stress Testing – Predicting Drug Degradation*, First Edition. New York: Taylor & Francis, 2005; 153: 205–34.
6. Felton LA, Yang J. A rapid technique to evaluate the oxidative stability of a model drug. *Drug Dev Ind Pharm* 2007; 33: 683–9.
7. Lombardo F, Campos G. How do we study oxidative chemical stability in discovery? Some ideas, trials, and outcomes. In: Borchard RT, Kerns EH, Lipinski CA, Thakker DR, Wang B, eds. *Pharmaceutical Profiling in Drug Discovery for Lead Selection*. Virginia: AAPS Press. 2004: 183–94.
8. Gamache P, McCarty J, Waraska J, Acworth J. Pharmaceutical oxidative stability profiling with high-throughput voltammetry. *American Lab* 2003; 35 (14): 21–5
9. Harmon PA, Kosuda K, Nelson E, Mowery M, Reed RA. A novel peroxy radical based oxidative stressing system for ranking oxidizability of drug substances. *J Pharm Sci* 2006; 95: 2014–28.
10. Sheldon RA, Kochi JK. Metal catalyzed oxidations of organic compounds in the liquid phase: a mechanistic approach. In: Eley DD, ed. *Advances in Catalysis*. New York: Academic Press, 1976: 273–413.
11. Ha E, Wang W, Wang J. Peroxide formation in polysorbate 80 and protein stability. *J Pharm Sci* 2002; 91: 2252–64.
12. Haung T, Garceau ME, Gao P. Liquid chromatographic determination of residual hydrogen peroxide in pharmaceutical excipients using platinum and wired enzyme electrodes. *J Pharm Bio Anal* 2003; 31: 1203–110.
13. Wasylaschuk WR, Harmon PA, Wagner G, et al. Evaluation of hydroperoxides in common pharmaceutical excipients. *J Pharm Sci* 2007; 96: 106–16.
14. Maillard B, Ingold KU, Scaiano JC. Rate constants for the reactions of free radicals with oxygen in solution. *J Am Chem Soc* 1983; 105: 5095–9.
- 15a. Denisov ET. *Handbook of Antioxidants*. Bond Dissociation Energies, Rate Constants, Activation Energies and Enthalpy of Reaction, 4th edn. Boca Raton, FL: CRC Press, 2000: 21–6
- 15b. McMillen, DF, Golden DM. Hydrocarbon Bond Dissociation Energies. *Ann Rev Phys Chem* 1982; 33: 493–532.
16. Foti MC. Antioxidant properties of phenols. *J Pharm Pharmacol* 2007; 59: 1673–85.
17. Carstensen JT. Drug stability: principles and practices. In: *Drugs and the Pharmaceutical Sciences*. New York: Marcel Dekker, 1990: 83–94.
18. Ingold KU. Peroxy radicals. *Acc. Chem. Res.* 1969; 2: 1–9.
19. Bateman L. Olefin oxidation. *Quart Revs* 1954; 8: 147–67.
20. Russel GA. Deuterium-isotope effects in the autoxidation of arylalkyl hydrocarbons. Mechanism of peroxy radicals. *J Am Chem Soc* 1957; 79: 3871–7.
21. Mayo FR. Free radical autoxidation of hydrocarbons. *Accounts Chem Res* 1968; 1: 193–201.
22. Anderson GH, Smith J. Acid catalyzed rearrangement of hydroperoxides. II. Phenylcycloalkyl hydroperoxides. *Can J Chem* 1968; 46: 1561–70.
23. Boccardi G, Deleuze C, Gachon M, Palmisano G, Vergnaud JP. Autoxidation of tetrazepam in tablets: Prediction of degradation impurities from the oxidative behaviour in solution. *J Pharm Sci* 1992; 81: 183–5.
24. Sosnovsky G, Zaret EH. Base catalyzed autoxidation. In: Swern D, ed. *Organic Peroxides*. New York: John Wiley and Sons, 1971: 517–60.
25. Russel GA, Bemis AG. The oxidation of carbanions: I oxidation of triaryl carbanions and other tertiary carbanions. *J Am Chem Soc* 1966; 88: 5491–7.
26. Gersmann HR, Bickel AF. Autoxidation of ketones and esters in basic solution. *J Chem Soc (B)* 1971; 11: 2230–7.
27. Gu L, Chiang H, Becker A. Kinetics and mechanisms of the autoxidation of ketorolac tromethamine in aqueous solution. *Int J Pharmaceutics* 1988; 41: 95–104.
28. Harmon PA, Biffar S, Pitzemberger SM, Reed RA. Mechanism of the solution oxidation of rofecoxib under alkaline conditions. *Pharm Res* 2005; 22: 1716–26.
29. Beaver BD, Cooney JV, Watkins JM Jr. Autoxidation of nitrogen heterocycles. 2. Kinetic measurements of the autoxidation of 2,5-dimethylpyrrole. *Heterocycles* 1985; 23: 2847–51.
30. Malhotra SK, Hostynek JJ, Lundin AF. Autoxidation of enamines and Schiff bases of α,β -unsaturated ketones. A new synthesis of unsaturated 1,4-diones. *J Am Chem Soc* 1968; 90: 6565–6.
31. Bartlett PD, Banavali R. Spontaneous oxygenation of cyclic olefins: effects of strain. *J Org Chem.* 1991; 56: 6043–50.

32. Seip M, Brauer H-D. Endoperoxide formation of helianthrene with triplet molecular oxygen. A spin-forbidden reaction. *J Am Chem Soc* 1992; 114: 4486–90.
33. Kawaguchi-Murakami Y, Fukutsu N, Kajiro T, et al. A prediction system of oxidation reaction as a solid state stress condition: applied to a pyrrole containing pharmaceutical compound. *J Pharm Biomed Analysis* 2009; 50: 328–35.
34. Brady Clark K, Howard JA, Oyler A. Retinoic acid oxidation at high oxygen pressure: evidence for spin-forbidden direct addition of triplet molecular oxygen. *J Am Chem Soc* 1997; 119: 9560–1.
- 35a. Howard JA, Ingold KU. Absolute rate constants for hydrocarbon autoxidation. I. Styrene. *Can J Chem* 1965; 43: 2729–36.
- 35b. Howard JA, Ingold KU. Absolute rate constants for hydrocarbon autoxidation. II Deutero systems and ring substituted systems. *Can J Chem* 1965; 43: 2737–43.
- 35c. Howard JA, Ingold KU. Absolute rate constants for hydrocarbon autoxidation. IV. Tetralin, cyclohexene, diphenylmethane, ethylbenzene and allylbenzene. *Can J Chem* 1966; 44: 1119–30.
- 35d. Howard JA, Ingold KU. Absolute rate constants for hydrocarbon autoxidation. VI. Alkyl aromatic and olefinic hydrocarbons. *Can J Chem* 1967; 44: 1113–18.
36. Hendry DG, Mill T, Piskiewicz L, Howard JA, Eigennam HK. A critical review of H-atom transfer in the liquid phase: chlorine atom, alkyl, trichloromethyl, alkoxy and alkylperoxy radicals. *J Phys Chem Ref Data* 1974; 3: 937–78.
37. Oyler AR, Naldi RE, Facchine KL, et al. Characterization of autoxidation products of the antifungal compounds econazole nitrate and miconazole nitrate. *Tetrahedron* 1991; 47: 6549–60.
38. Boccardi G. Autoxidation of drugs: Prediction of degradation impurities from results of reactions with radical chain initiators. *Il Farmaco* 1994; 49: 431–5.
39. Alsante KM, Martin L, Baertschi SW. A stress testing benchmarking study. *Pharm Technol* 2003; 27: 60–72.
40. Nelson ED, Thompson GM, Yao Y, Flanagan HM, Harmon PA. Solvent effects on the AIBN forced degradation of Cumene: implications for forced degradation studies. *J Pharm Sci* 2009; 98: 959–69.
- 41a. Watkins MA, Templeton AC, Harmon PA. Structure and mechanism elucidation of non-characteristic forced degradation reaction products from an azonitrile radical initiator. Oral presentation at the Institute for International Research 3rd Annual Conference on Forced Degradation Studies, Short Hills, NJ, February 28, 2006.
- 41b. Watkins MA, Harmon PA. Identification and elimination of alkoxy radical mediated degradation during azo nitrile initiated oxidative stressing. Manuscript in preparation.
42. Talet-Erben T, Onol N. The reaction of 2 cyano-2 propyl free radicals with oxygen. *Ca J Chem* 1960; 38: 1154–7.
43. Nelson ED, Harmon PA, Szymanik RC, et al. Evaluation of solution oxygenation requirements for azonitrile based oxidative forced degradation studies of pharmaceutical compounds. *J Pharm Sci* 2006; 95: 1527–39.
44. Harmon PA, Kosuda KM, Nelson ED, Mowery MA, Reed RA. A novel peroxy radical based oxidative stressing system for predicting oxidative instability of active pharmaceutical ingredients. *J Pharm Sci* 2006; 95: 2014–28.
45. Bach RD, Ayala PY, Schlegel HB. A reassessment of the bond energies of peroxides: an ab-initio study. *J Am Chem Soc* 1996; 118: 12758–65.
46. Payne GB, Deming PH, Williams PH. Reactions of hydrogen peroxide VII. Alkali-catalyzed epoxidation and oxidation using nitrile as co-reactant. *J Org Chem* 1961; 26: 659–63.
47. Laus G. Kinetics of acetonitrile assisted oxidation of tertiary amines by hydrogen peroxide. *J Chem Soc Perkins Tans* 2001; 2: 864–8.
48. Nakamura T, Maeda H. A simple assay for lipid hydroperoxides based on triphenylphosphine oxidation and high performance liquid chromatography. *Lipids* 1991; 26: 765–8.

7 | Photostability stress testing

Elisa Fasani and Angelo Albini

INTRODUCTION

The problem of photostability of drugs has received relatively little attention in the pharmaceutical industry. Furthermore, until a decade ago, the degradation studies carried out involved variable protocols, every single issue being confronted in a unique manner by the various laboratories involved. This contrasts with the general consensus in the determination of drug stability in other respects (e.g., thermal and moisture). In the case of photostability, there are many characteristic variables that can be chosen among a large number of possibilities, such as the light source, the time of exposure, the sample presentation (a determining factor since light must be adsorbed to cause an effect, and this depends on the physical state). As an example, the light source used for photodegradation studies in the pharmaceutical industry has varied from lamps emitting only or mainly in the UV, including UV-C, a choice clearly unrelated to “in use” conditions, to windowsill exposure to window-glass filtered daylight. As a consequence, data from different laboratories could not be readily compared.

In 1998, the implementation of the ICH guidelines as how to carry out photostability tests on new pharmaceutical drug substances and drug products (ICH Q1B, adopted and published in the Code of Federal Regulations in May 1997) (1–3), introduced a reference point. This guideline is available as an annex to the *ICH Guideline for Stability Testing of New Drug Substances and Drug Products* (4). Thus, there are now official guidelines to which the pharmaceutical industry should refer, and in addition, a general information chapter on photostability testing was recently published in the *United States Pharmacopeia* (5).

The ICH guideline mainly concerns the determination of the photostability of drug substances and manufactured finished drug products, though not specifically addressing the problem of parameterizing “in use” conditions.

However, as has been discussed in several instances (6–17), the guideline leaves much to be desired in terms of scientific exactness (e.g., the suggested actinometer has several disadvantages) as well as of univocal practical interpretation (e.g., two possibilities are presented for the light source, “Option 1 and Option 2,” but it is not clear whether these are equally appropriate).

The guideline refers to confirmatory testing, where a fixed light dose impinges on the sample. Such studies are analogous to accelerated stability studies, as defined in the parent guideline. It also hints to the possibility of carrying out stress testing. The latter is a scientific investigation into the “intrinsic stability characteristics” of the molecule, and is used as a predictor of potential photostability concerns. Thus, stress testing is analogous to forced degradation studies and, in contrast to confirmatory studies, is not part of the formal definitive stability program. Therefore, this is not a highly regulated program, and is meant to provide suitable information to develop and validate test methods for the confirmatory studies and to gain stability mechanistic understanding. It should establish photodegradation pathways and allow identification of degradation products in order to validate the fitness of the analytical procedures used for confirmatory studies. Therefore, as is general for stress testing (4), photochemical stress testing is usually carried out under more severe conditions than those chosen for the accelerated test procedures.

In order to facilitate carrying out such tests, some background information about the course and the main photochemical reactions is supplied in the following section.

PHOTOCHEMISTRY OF DRUGS

Photochemical Reactions

Photochemical reactions involve electronically excited states which are formed through absorption of ultraviolet or visible light by molecules. As the monodimensional (Jablonski) diagram in Figure 1 shows, the promotion of an electron from an occupied molecular orbital (e.g., from

the highest occupied molecular orbital, the HOMO) to one of the upper (more energetic) unoccupied molecular orbitals (e.g., the lowest one, the LUMO), caused by the absorption of photons of appropriate frequencies, leads to the formation of a variety of excited states. The absorption events represented in Figure 1 are called “vertical” transitions, because they occur in a time domain shorter than that required for atomic vibrations to take place. Contrary to the ground state, excited states are open-shell species (two singly occupied orbitals) and therefore every electronic configuration gives rise to two states of different energy, a singlet (no net total spin) and a triplet (total spin=1) state. For every configuration the triplet is lower in energy than the singlet. Spin inversion during absorption is forbidden and therefore light absorption by the ground state (S_0) leads only to singlet excited states. $S_0 \rightarrow S_n$ transitions appears as bands of different intensity in the absorption spectrum. This is due to the different probability of each electronic transition, measured by the molar absorptivity coefficient, ϵ , in the Lambert–Beer equation $A = \log(1/T) = \epsilon bc$, where b is the optical path and c the concentration. Because $S_0 \rightarrow T_n$ transitions have negligible probability to occur, the respective bands are not observed in electronic spectroscopy (a very long optical path would be required). However, triplet states may be reached through an indirect path, via a first-formed electronically excited singlet state (either the S_1 state or an upper S_n state). The latter process is known as inter-system crossing (a “horizontal”—no energy change involved—process wherein the spin is inverted) and in some cases it occurs with a high (up to unitary) probability.

The wide absorption bands in the spectrum of molecules are due to the formation of electronically excited states with additional vibrational quanta. After excitation, however, the excess energy within each state is rapidly lost (via vibrational relaxation). Furthermore, conversion from any upper (S_n or T_n) to the lowest lying (S_1 or T_1) electronically excited state of the same multiplicity (internal conversion, again a horizontal transition) is very fast (typically $>10^{12} \text{ s}^{-1}$). Indeed, in almost every case, such relaxation processes are faster than any other chemical (a reaction) or physical (light emission) process. The low-lying states, though still quite short-lived (typical lifetimes are $\leq 10^{-8} \text{ s}$ for S_1 and $\leq 10^{-6} \text{ s}$ for T_1), do have the time for undergoing a reaction or emitting light. Summing up, for all practical purposes only two excited states need to be considered along with ground state S_0 : that is, the first singlet (S_1) and the first triplet (T_1) states (this is the so-called rule of three states).

Clearly, this holds for molecules containing a single chromophore (see section “The Chromophore”). When two independent chromophores are present, excitation at the wavelength where one or the other (mainly) absorbs may lead to a different chemistry. An example is shown below in Figure 6 for a glucocorticoid where both a cross conjugated ketone and an isolated ketone are present. Irradiation at either 254 or 366 nm excites mainly the former and causes the typical rearrangement, while the other chromophore is mainly excited at 310 nm and α -cleavage of the isolated ketone takes place.

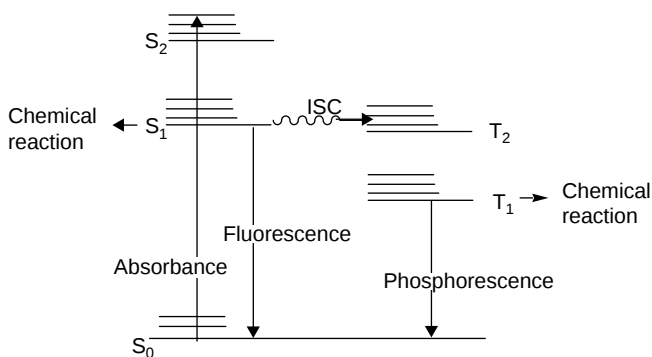


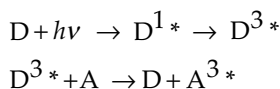
Figure 1 Jablonski diagram.

The main practical consequences of the above generalizations are (i) that light needs to be adsorbed in order to cause a chemical effect and (ii) that the photoreaction (or light emission) in most cases does not depend on the wavelength of the light used. The "action spectrum" is the rate of a photochemical effect plotted against wavelength of light, that is the relative effectiveness of different wavelengths of light in causing any kind of photochemical action. Since, as indicated above, the photochemistry generally remains the same whichever is the exciting wavelength (at least in solution), the "action spectrum" corresponds to the absorption spectrum of the "active" molecule and under proper conditions (typically at a low enough concentration) is superimposable to it. This holds for a single light-absorbing species since when different species are present (the simplest instance being that of an acid or basic compound in different ionization states) the fraction of light absorbed by each species changes with the wavelength and so does the chemistry observed.

A related concept that identifies the spectral regions of the source used and has more an applied than a scientific significance is that of "activation spectrum." This empirically refers to the relative amount of degradation caused by a specific wavelength on a given material that is responsible for changes in appearance and/or properties of the material. As such, this is affected by the presence of impurities, by any inhomogeneity of absorbance through the layer of the material, by the type of degradation measured and, as a result of the previous factors, by the type of light source used.

Also, it is important to know whether the products from the initial photochemical reaction absorb light in the wavelength interval used. If these photoproducts are transparent, light is absorbed only by the reactant and the photoreaction proceeds undisturbed up to complete reagent consumption. If, as quite commonly observed, this is not the case, competitive absorption from the products increases during the photochemical reaction ("inner filter" effect) and the photoreaction slows down progressively, changes its course, or, when the primary products are photoreactive themselves, leads to an increasingly complicated mixture including secondary photoproducts.

Furthermore, it is important to note that electronic excitation can be transferred from an excited state D^* to a ground state molecule A (this is known as sensitization of A by energy donor D and is a very fast process, provided that A has some excited state lower than D^*). This is a way of arriving at the triplet of a molecule for which the intersystem crossing is too slow. Thus, a molecule (e.g., molecule A) transparent to the wavelength range used may arrive at the triplet states through energy transfer from another light-absorbing molecule D, provided that the latter has a high enough triplet and an efficient ISC:



As a result, a nonabsorbing molecule A reacts and D acts as sensitizer. When different molecules are present in solution and absorb competitively, the population of the excited states depends not only on the relative absorption at each wavelength, but also on such energy transfer processes. Therefore, while in a simple system with a single-absorbing molecule, all photochemical and photophysical effects do not depend on the wavelength used, the overall result in a complex system often depends on the exciting λ . This is often the case for drug preparations (see section "Photochemistry of Drug Preparations").

Although this may seem unnecessarily complicated at first sight, it is important to distinguish the primary photochemical event (the one proceeding directly from the excited state) from the overall chemical process. Excited states lie quite high in energy and often lead to products that are unstable at room temperature. Highly strained (though ground state) molecules as well as unstable "intermediates," such as radicals, ions, carbenes, nitrenes, etc., may be formed from photochemical reactions. As an example, 1-phenylcyclohexene undergoes *Z-E* isomerization in the excited state, but the highly strained *E* isomer reverts to the *Z* form in microseconds

(Fig. 2, path a), so that, although its formation may be demonstrated, no net chemistry results (at least at room temperature), despite the occurring of an efficient excited state reaction (18). However, in the presence of a protic solvent, an addition to the strained *E*-cycloalkene occurs, and this highly energetic molecule reacts irreversibly, rather than reverting to the starting *Z*-isomer (Fig. 2, path b) (19). In another example, excited benzophenone abstracts a hydrogen atom from *iso*-propanol, yielding α -hydroxy radicals. The following reaction of such species depends on conditions and may be driven to give either benzopinacol or benzhydrol by adding a base, which affects the chemistry of the radicals, not that of the excited state (Fig. 3) (20).

In the example above, the photodegradation product profile results from subsequent thermal reactions of the primarily formed intermediates, rather than from a direct effect on the formation and reactivity of the excited state, and this is often the case. On the other hand, when a change in the medium changes the nature of a molecular orbital—e.g., by protonation of a nonbonding orbital—or deeply affects its energy—e.g., by establishing a strong hydrogen bond, the electronic structure of the excited state also changes and so do the photochemical reactions. As a typical example, the lowest triplet state of many ketones is an $n\pi^*$ state and this triplet state abstracts hydrogen efficiently. A protic medium may sufficiently stabilize the n_O orbital to bring the $n\pi^*$ state above the $\pi\pi^*$ state. The $\pi\pi^*$ state thus becomes the lowest excited state and is, therefore, the reactive one. As a result, some ketones are much slower at hydrogen abstraction in protic than in aprotic media, and therefore the photodegradation profiles can be significantly different depending upon the solvent system.

Some further important peculiarities of photochemical reactions should be stressed even in a rudimentary introduction. As an example, photochemical reactions are expected to depend little on the temperature. This is because excited states have a short lifetime and thus only very fast reactions (with a very low activation energy, in the order of a few kcal M^{-1} , and thus next to negligible temperature effect on the rate) may compete with decay to the ground state.

Furthermore, contrary to the case of a thermal reaction, the order of a photochemical reaction cannot be defined, since the observed rate of reaction depends on the rate of formation of the excited state, i.e., on the rate of *light absorption* by the reacting molecule. This is not directly related to either the substrate concentration $[S]$ or the light flux. If light is absorbed efficiently

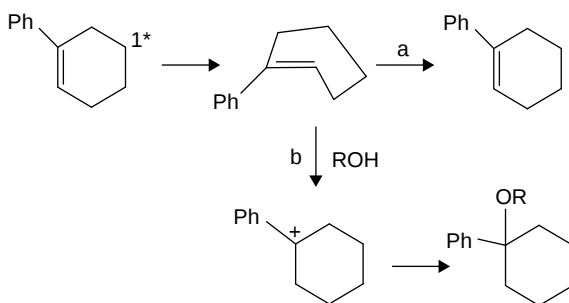


Figure 2 Photoinduced isomerization (path a) and addition (path b) of phenylcyclohexene.

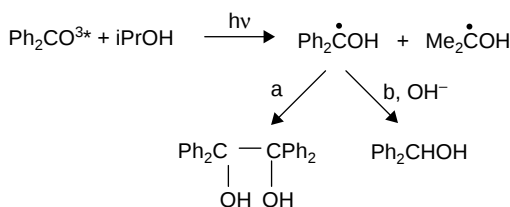


Figure 3 Photoinduced hydrogen abstraction by benzophenone.

(viz., absorbance A remains ≥ 1 throughout the experiment, a quite common occurrence taking into account that ϵ_{max} is 10^2 to $>10^4$ for most organic molecules), then the fraction of light absorbed does not change appreciably up to a high conversion, e.g., 90%; in this case, the observed rate of reaction remains constant (apparent zero order):

$$-d[S]/dt = k$$

On the other hand if the rate of absorption changes, whether because the fraction of light absorbed by the active molecule changes during the conversion (due to competitive absorption by the products or another interference or because of the participation of sensitization processes), the overall rate changes and the observed apparent order may change during the conversion. Another limiting case should be mentioned, although perhaps less frequently encountered in photostability studies, is that of poorly absorbing solutions, namely either very dilute ones or those for which the absorptivity of the substrate is low at the λ used. In this case, the decomposition often appears to occur according to a first order rate. This is because the absorbance A , and thus the rate of excitation, varies linearly with the substrate concentration for small values (e.g., $A < 0.01$):

$$-d[S]/dt = k[S]$$

Carrying Out a Photochemical Reaction

The first precaution when carrying out a photochemical reaction is ensuring that light reaches the target molecule. Trivial as it may seem, this point is sometimes forgotten. Thus, the emission of the lamp and the absorption band of the substrate should contain some overlap, and no components of the system (the lamp envelope, a cooling system, the vessel containing the substrate, and the solvent) should absorb competitively. It should also be ensured that a sufficient fraction of the lamp emission reaches the sample rather than being dispersed in other directions. This is easy when the lamp output is focused onto the sample by mirrors or lenses or when the lamp is immersed in the solution to be irradiated, but may not be so easy when external lamps are used.

The effect of the medium and of impurities on the course of a photochemical reaction is quite different from what is usually observed with thermal reactions. In a way, photochemical reactions may be considered minimally affected by many experimental parameters, because reactions of excited states are so fast. On the other hand, some impurities may specifically interact with the excited state at a very high (diffusion controlled, $>1 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$) rate. A typical such case is that of compounds having low-lying triplets, which can function, as illustrated above, as acceptors in an energy transfer process and thus "quench" the photoreaction. In that case, a low amount of an impurity may effectively suppress a photoreaction. Typical examples are dienes or polyenes often present in organic solvents and, above all, oxygen, the lowest-energy transition of which is lower than that of the excited states of practically every organic molecule. Therefore, it is recommended that one of the conditions under which photochemical tests are carried out involves "deoxygenation" by flushing for some minutes with an inert gas such as nitrogen or argon (in the case of a solution) or by having the sample in an inert atmosphere (in the case of solids). This is sufficient for making oxygen quenching of excited states insignificant. Such experiments can be compared with nondeoxygenated tests. This is important because it is possible for oxygen to intervene at different stages of the reaction, in addition to quenching the excited state. As an example, it may easily trap radicals formed in the course of the reaction.

Classification and Examples

The Chromophore

Spectroscopists are accustomed to classify molecules according to the chromophore they contain, namely, the moiety to which the absorption(s) observed can be attributed. In terms of pharmaceutical photostability testing, absorption in the near UV (UV-B, 280-315, UV-A,

315–400 nm) or in the visible is of the greatest concern since this is the range of wavelengths present in indoor or outdoor light. Absorptions in these regions mostly involves $n \rightarrow \pi^*$ or $\pi \rightarrow \pi^*$ transitions and thus correspond to (conjugated) carbon–carbon or carbon–heteroatom multiple bonds or to aromatic systems. Recognizing the relevant chromophore(s) is immediately relevant also for the photochemistry since this identifies the type and localization of the excited state involved. In fact, photochemical reactions are usually classified according to the chromophore present in the molecule and are rationalized on the basis of the modification of the electronic structure occurring upon excitation (21–23). The chromophores to which photochemical reactivity is usually connected has been previously reviewed, with specific examples concerning the photochemistry of drugs (9,24).

Ketones

The lowest-lying excited state of ketones often correspond to a $n_{\text{O}} \rightarrow \pi_{\text{C=O}}^*$ transition (λ_{max} for aliphatic derivatives is around 280 nm range or further to the red for conjugated or aryl derivatives) and the chemistry of such species is determined by the presence of an unpaired electron on the n_{O} orbital. This imparts a radical and electrophilic character to such species, which closely parallels that of alkoxy radicals. Thus, a typical reaction is intermolecular hydrogen abstraction (finally resulting in reduction of the ketone function). Intramolecular abstraction is also fast and usually involves the easily accessible γ position. The latter reaction yields a 1,4-biradical that evolves through $\text{C}_{\alpha}-\text{C}_{\beta}$ bond cleavage ("Norrish type II" reaction) or through cyclization to give a hydroxycyclobutane (Yang reaction) (Fig. 4). Relatively weak bonds in the α -position may undergo homolytic cleavage, another reaction reminiscent of the behavior of alkoxy radicals, as exemplified in the case of metyrapone (Norrish type I reaction, Fig. 5) (25).

Conjugated ketones are mostly poor hydrogen abstractors. These may undergo rearrangement (quite common among glucocorticosteroids, Fig. 6) (26,27), addition reactions, and

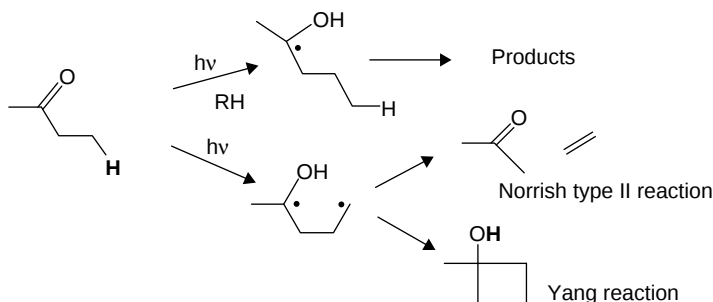


Figure 4 Inter- and intramolecular hydrogen abstraction.

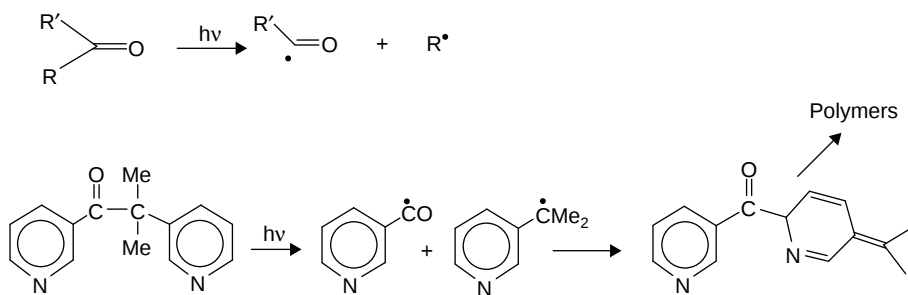


Figure 5 Norrish type I reaction: photochemistry of metyrapone.

2+2 cycloaddition reactions. The last class of reactions has been often observed by irradiation in the solid state with conjugated ketones (28). α -Santhoinin, the first drug formulated in the United States and long known for its photolability, offers a nice example of a contrasting behavior in solution versus in the crystal state (29) (Fig. 7).

Nitro Compounds

Nitro compounds, in particular aromatic and heterocyclic derivatives, absorb strongly in the near UV and are in many senses similar to ketones in the properties of their excited states, again characterized by an unpaired electron in the n_o orbital and thus by a radical character. A typical example is that of the easy intramolecular hydrogen abstraction in nifedipine and related vasodilators (Fig. 8) (30). Another manifestation of the radical character of the nitro group is the

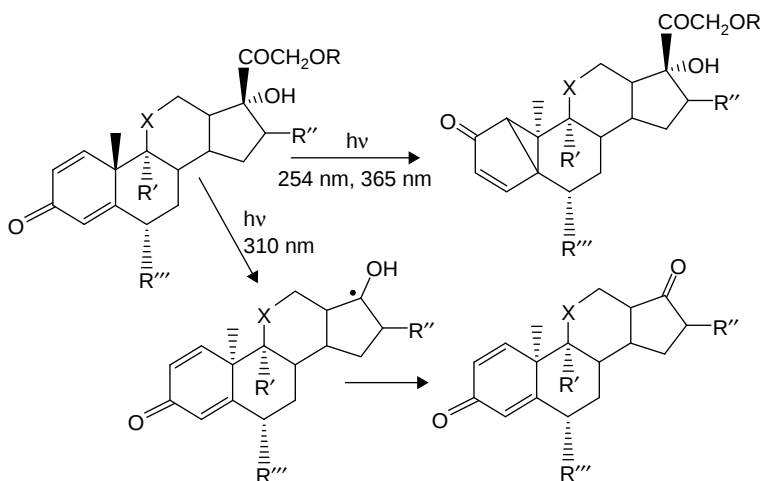


Figure 6 Photochemistry of some glucocorticosteroids: dependence on the exciting wavelength.

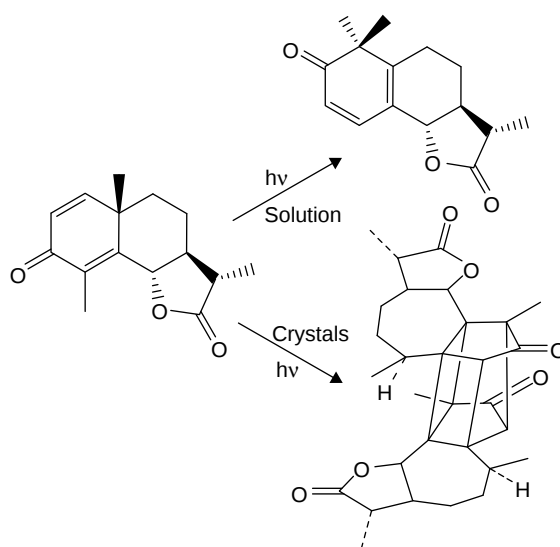


Figure 7 Photoreaction in solution versus solid state: photochemistry of α -santhoinin.

rearrangement often observed with nitrated five-membered heterocycles, as in the case with metronidazole (Fig. 9) (31).

Alkenes

Nonconjugated alkenes do not absorb in the near UV, but polyenes or arylalkenes absorb strongly in the UV and, for extended conjugation, in the visible region. Furthermore, these derivatives have low-lying triplet states that can be populated by sensitizers (including those

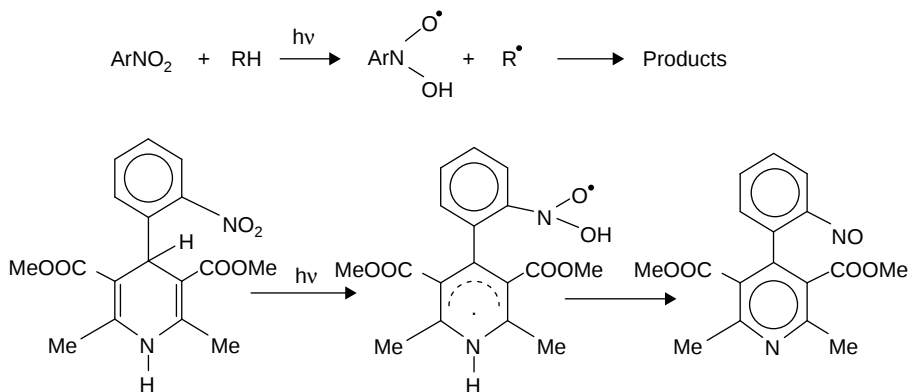


Figure 8 Photochemistry of nitro compounds: the case of nifedipine.

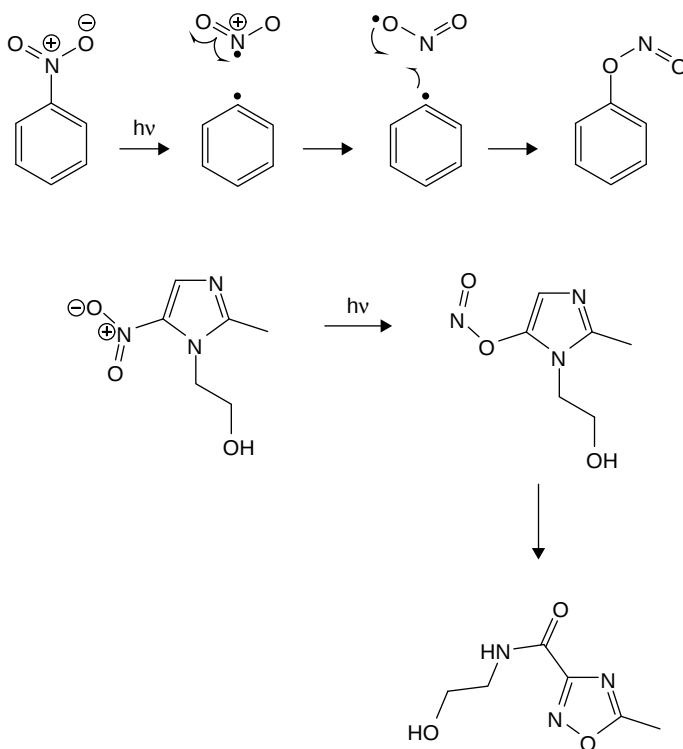


Figure 9 Rearrangement of nitro group: photochemistry of metronidazole.

adventitiously present). In the excited $\pi\pi^*$ state, the double bond is broken and alkenes are invariably characterized by efficient *E-Z* isomerism in the excited state, except when torsion is hindered by molecular constraint. Typical examples are synthetic estrogens (Fig. 10) (32) or vitamin A (33). Conjugated polyenes may also undergo electrocyclic reactions of the hexatriene-cyclohexadiene or of the butadiene-cyclobutene type, as observed with the above estrogens (32) or with vitamin D derivatives (35,36). Atorvastatin (34), where the heterocyclic ring hinders isomerization, undergoes only cyclization (Fig. 11). Photochemical *E-Z* isomerism is not unique to the C=C bond; indeed, it is general with practically every A=B bond, e.g., azo compounds, oximes (37–39), hydrazones (40), etc. Another path for alkenes, characteristic of the electron-rich derivatives, is hydration, as observed with the methylenedibenzothiazine moiety in chlorprothixene (Fig. 12) (41).

Aromatic and Heterocyclic Derivatives

These compounds are well known for the strong absorption in the UV as well as, for higher members of the series, in the visible. The large stabilization connected to aromaticity extends also to excited states, and aromatic and heteroaromatic compounds often decay through a physical path (typically by re-emission of a photon, viz. fluorescence or phosphorescence) rather than through a chemical path. However, significant and even efficient photoreactions are known for several derivatives. Such is the case for halogenated compounds. Cleavage of the

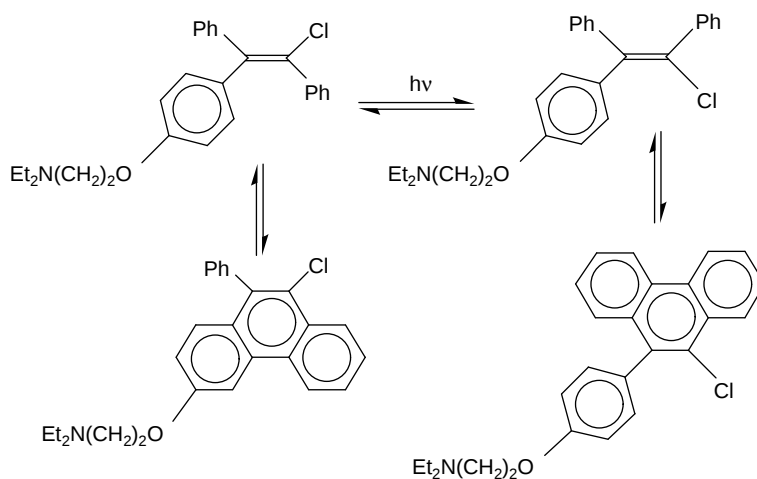


Figure 10 *E-Z* isomerism and electrocyclic reaction in the photochemistry of alkenes: the case of clomiphene.

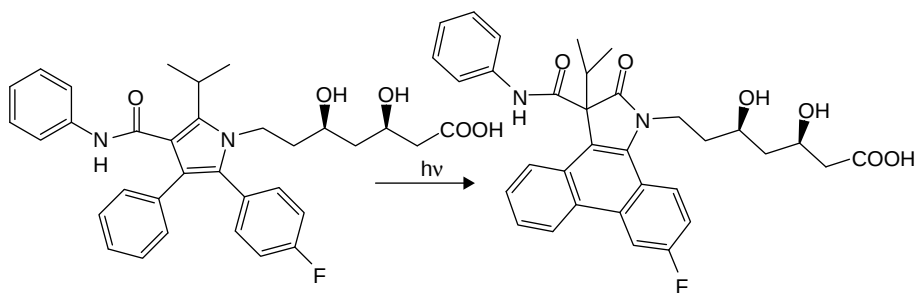


Figure 11 Atorvastatin electrocyclic reaction.

C–X bond may occur as a homolytic process, usually with low quantum yield for aryl chlorides (42) and more efficiently for bromides and iodides (Fig. 13) (43), or as a heterolytic process. The latter course is followed, in some cases with a high quantum yield, by some (hetero)arylfluorides, notably fluoroquinolones and oxazolidinone antibacterials. Irradiation in water causes fluoride liberation and, depending on the structure, either substitution by a hydroxyl group (Fig. 14) or reduction (Fig. 15). Defluorination may be accompanied by a further transformation. In fluoroquinolones, when a second fluorine atom is present at position 8, this is preferentially lost and the subsequently generated aryl cation undergoes an intramolecular attack onto the *N*-alkyl chain (Fig. 16) (44–46). Another general photocleavage involves sulfa drugs that are usually susceptible to homolytic fragmentation of the C–SO₂ bond (Fig. 17) (47).

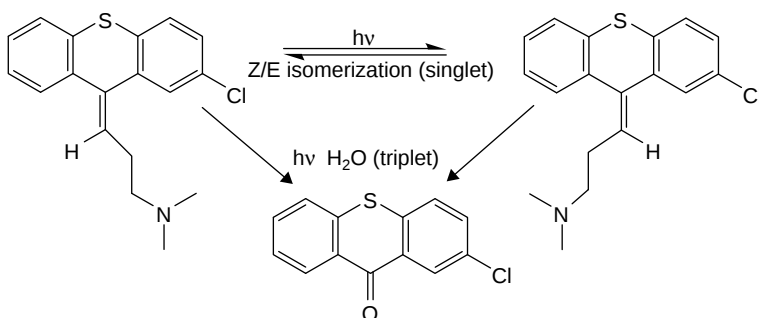


Figure 12 Photochemistry of chlorprothixene.

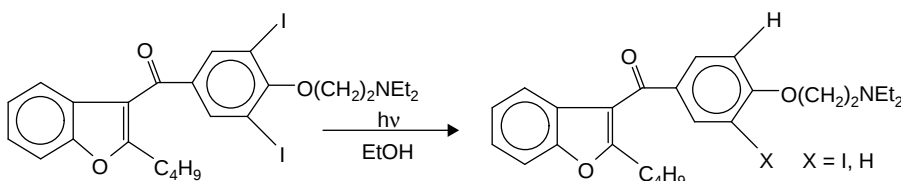


Figure 13 Dehalogenation: homolytic C–I bond cleavage in amiodarone.

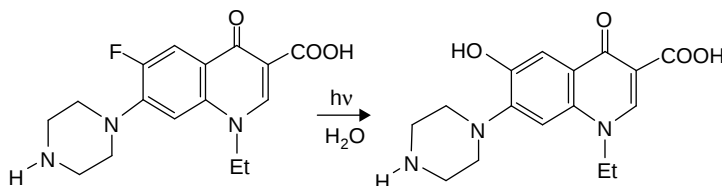


Figure 14 Heterolytic cleavage of C–X bond: substitution by a hydroxyl group in fluoroquinolones.

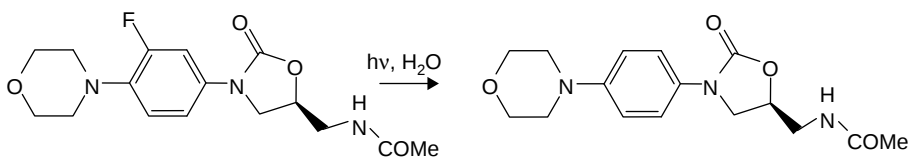


Figure 15 Heterolytic cleavage of C–X bond: reduction in oxazolidinones.

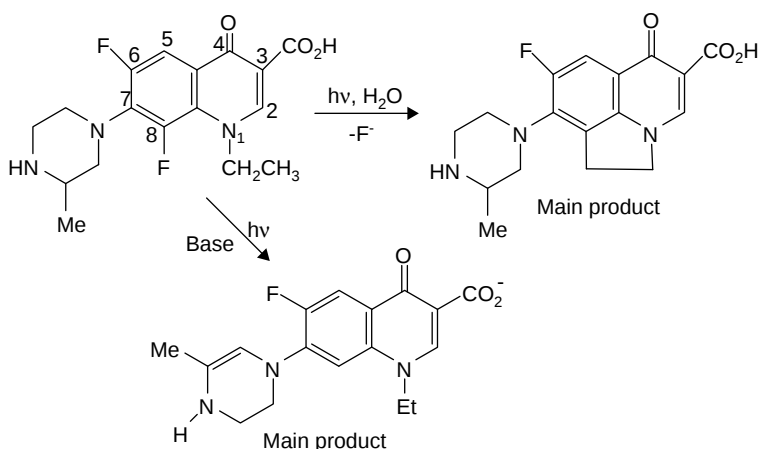


Figure 16 Photochemistry of difluorinated fluoroquinolones.

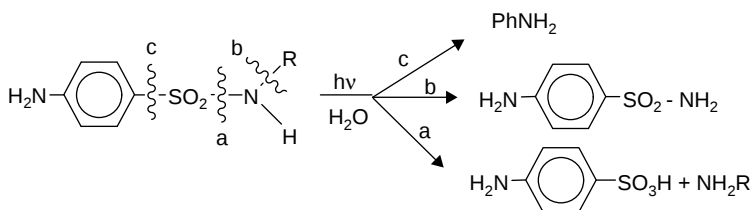


Figure 17 Photocleavage of C-SO₂ bond in sulfa drugs.

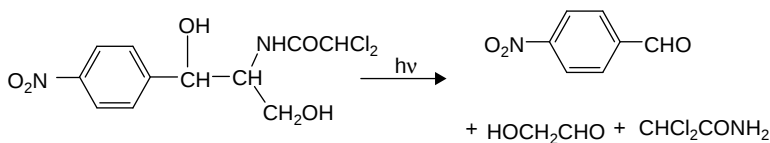


Figure 18 Cleavage at the benzylic position in chloramphenicol.

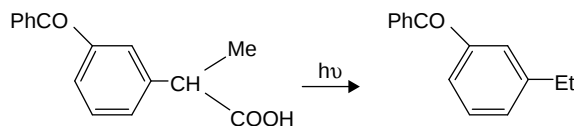


Figure 19 Decarboxylation of arylpropionic acid.

Cleavage at the benzylic position has been observed in a variety of (hetero)aromatic derivatives such as chloramphenicol (Fig. 18) (48) or methotrexate (49). An important case is that of the decarboxylation of arylacetic or 2-arylpropionic acids used as nonsteroidal antiinflammatory agents (Fig. 19) (50–55). Aryl alkyl ethers likewise tend to fragment at the ArO–C bond (56).

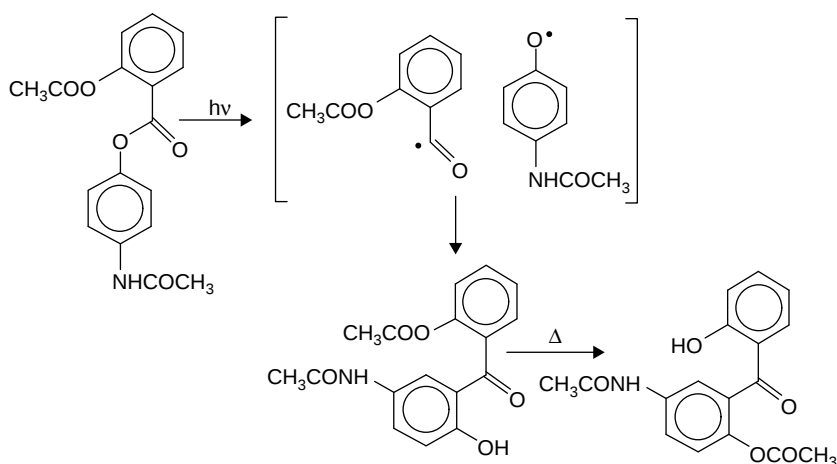


Figure 20 Fries-type rearrangement.

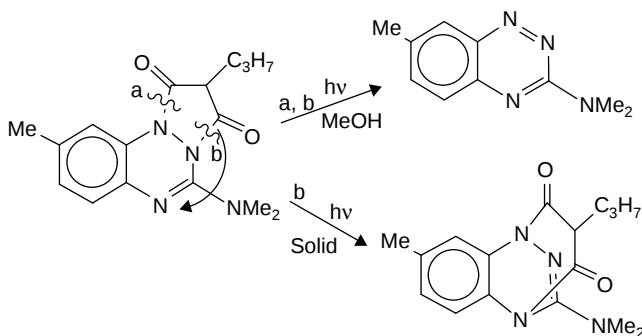
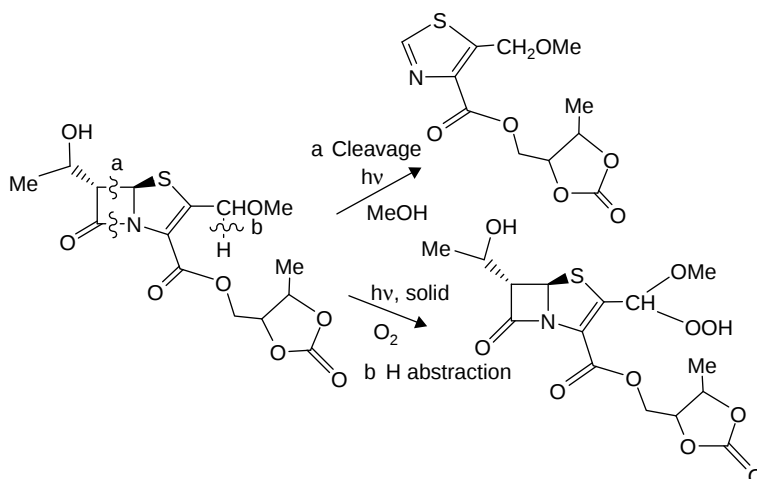
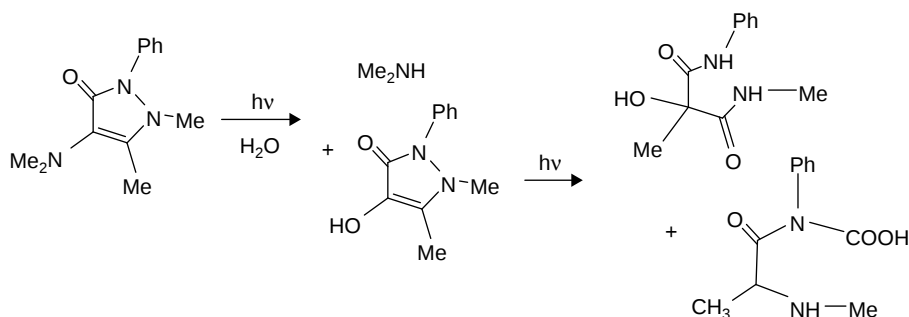
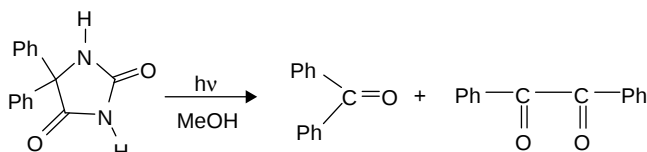


Figure 21 Photochemistry of azapropazone.

Fries-type rearrangements (Fig. 20) (57) also occur photochemically (this is generally a homolytic process, in contrast to the ionic mechanism of the thermal process). Azapropazone undergoes photoinduced 1,3-acyl shift in the solid state and fragmentation in solution (Fig. 21) (58).

Heterocycles are even more common than their carbocyclic analogs among drugs, and photoreactivity is a frequent occurrence. Strained rings often fragment, as it has been shown to be the case with the four-membered ring in a penem (Fig. 22, note, here as in the case of azapropazone above, the difference between the reaction in solution and in the solid state) (59). For both five- and seven-membered rings either photo-induced rearrangement or fragmentation are often observed, as exemplified by the photocleavage of pyrazolones (used as analgesic) (Fig. 23) (60–62) and imidazolones (e.g., phenyltoin, Fig. 24) (63). Benzodiazepines (see e.g., alprazolam in Fig. 25) generally are photolabile and undergo ring contraction and ring cleavage (64–68). Six-membered heterocycles are generally more stable, though there are exceptions as shown by the fragmentation observed with many barbituric acid derivatives (see Fig. 26 for barbital) (69–71). A class that is invariably characterized by photochemical reactivity is that of *N*-oxides (e.g., minoxidil (72) and clordiazepoxide (66)). Photocycloadditions may occur with suitable structures. As an example, the irradiation of trazodone under dilute conditions seems

**Figure 22** Photolysis of a penem.**Figure 23** Photofragmentation of pyrazolones.**Figure 24** Photofragmentation of imidazolone.

to lead to both [2+2] cycloaddition and [4+4] cycloaddition followed by a second cycloaddition to give a cage compound (Fig. 27) (73).

Oxidations

Oxidation processes are a common occurrence upon irradiation, in particular, in the presence of oxygen. Oxygen dissolved in solution is "activated" through different sensitization processes (take into account that the role of sensitizer may be exerted by the substrate itself, by a cofor-mulant or by an impurity). Many dyes are efficient oxygen sensitizers and due to the strong absorptivity are effective even at low concentrations. As far as the mechanism is concerned,

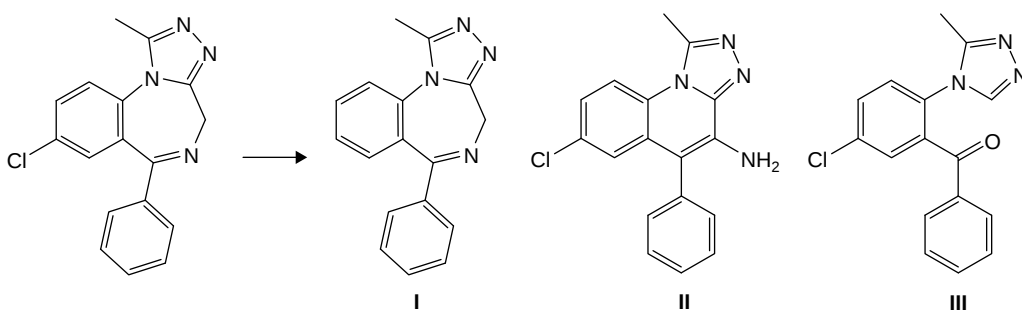


Figure 25 Photo-induced ring rearrangement of alprazolam.

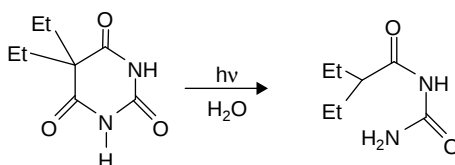


Figure 26 Photo-fragmentation of barbituric acid.

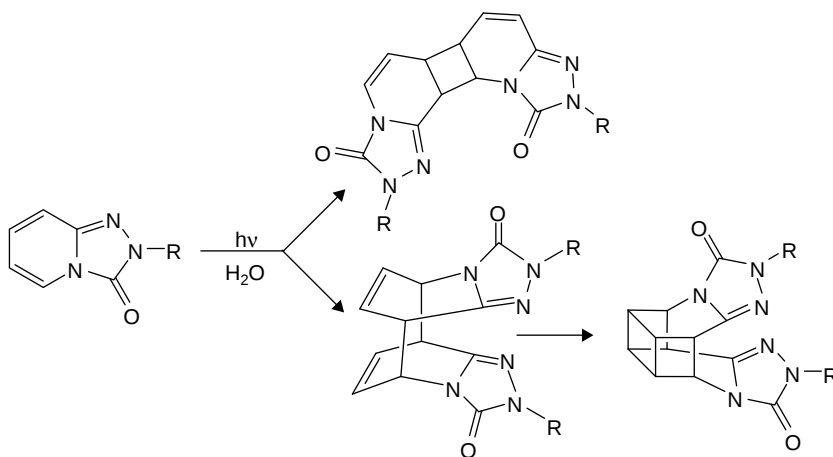
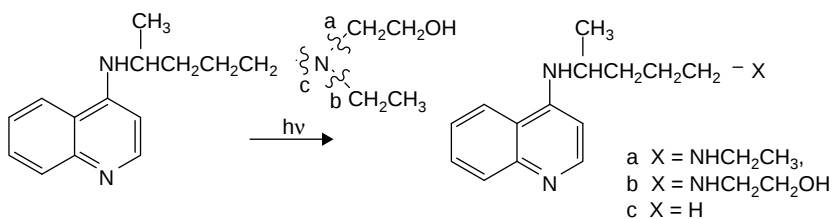
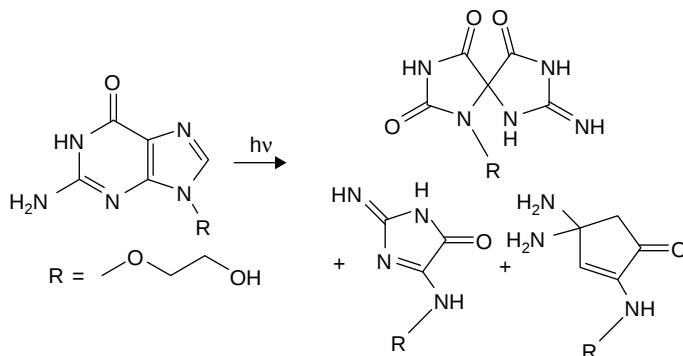
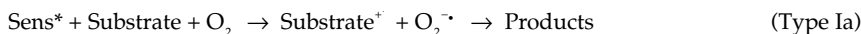
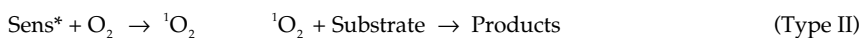
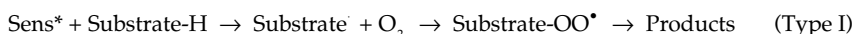
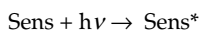


Figure 27 Irradiation of trazodone under dilute conditions.

oxygenations are classed in three groups (74). In type I oxygenations, the sensitizer interacts first with the organic substrate, e.g. leading to an alkyl radical. This radical is then trapped by ground state oxygen to give a peroxy radical that then further evolves to the end products. In type II oxygenations, the sensitizer first interacts with oxygen promoting it to the singlet state (remember that oxygen has a triplet ground state and excited singlet states). The energy of the lowest-lying singlet is a mere 22.4 kcal/mol, and thus, energy transfer from excited states of practically every organic molecule is possible (see above). Singlet oxygen is a powerful electrophile. For example, it reacts easily with alkenes undergoing an ene reaction or, with electron-rich

**Figure 28** Photodegradation of hydroxychloroquine.**Figure 29** Photochemistry of acyclovir.

derivatives, a [2+2] cycloaddition. A further mechanism for oxygenation sensitization involves electron rather than energy transfer; the electron transfer yields the radical cation of the substrate and superoxide anion and one or both of these species is involved in the subsequent reactions (Type Ia):



Summing up, moderate and good nucleophiles as well as H donors often undergo oxygenation, dehydrogenation, or oxidative fragmentation upon irradiation in the presence of oxygen. This is the case for amines (e.g., hydroxychloroquine in Fig. 28) (75), heterocycles (e.g., acyclovir, Fig. 29, and melatonin) (76,77), phenols, e.g., adrenaline (78) and some gonadotropic steroids (79), as well as for less good donors such as aromatic hydrocarbons (preferentially reacting at the benzylic position if a side chain is present, e.g., trimethoprim, Fig. 30) (80) and alkenes (Fig. 31) (81). Sulfides are easily oxidized to sulfones and/or sulfoxides, as observed e.g., with levomepromazine (Fig. 32) (82), dothiepin (83) and with phenothiazines (84). As shown in the figure, S-oxidation prevails over N-oxidation with levopromazine. Both ring sulfur and side-chain amino nitrogen are oxidized in cyamemazine (Fig. 33) (85).

Inorganic Compounds

Metal complexes are often colored and usually prone to photoinduced ligand exchange, as exemplified by the well-known cases of nitroprussiate (86) and cisplatin and analogs (Fig. 34) (87,88).

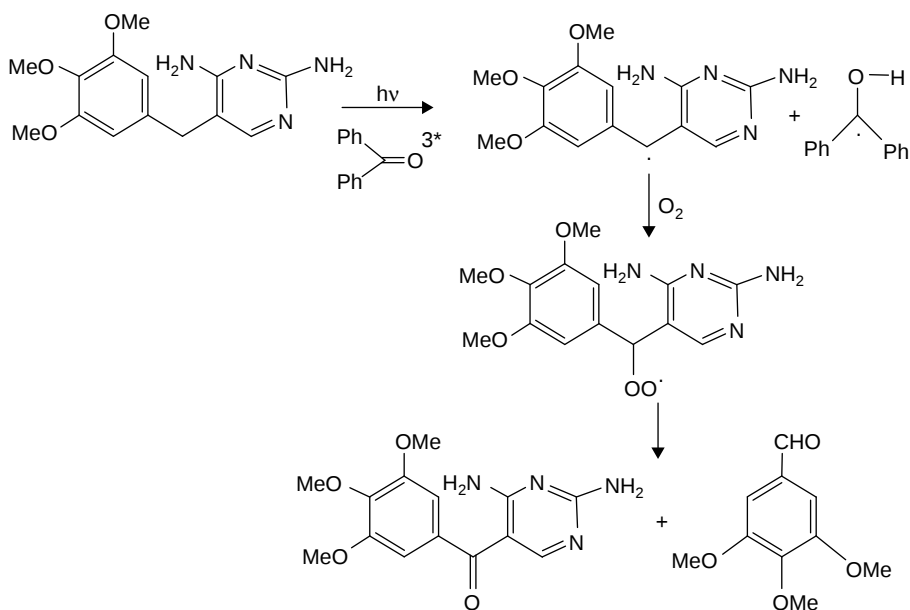


Figure 30 Photochemistry of trimethoprim.

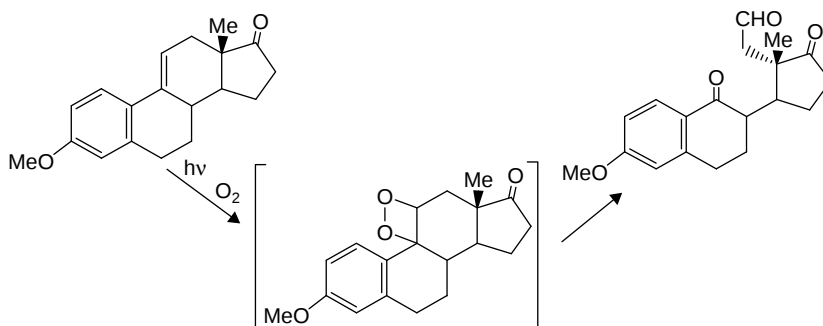


Figure 31 Oxidative photofragmentation of a C=C bond.

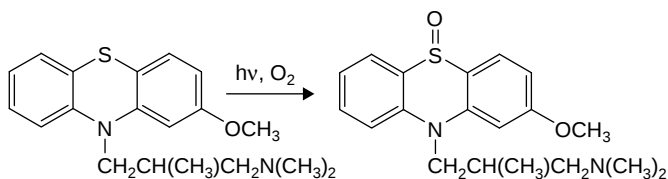


Figure 32 Photooxidation of the sulfide function in levomepromazine.

Biomolecules

Many drugs are biomolecules (i.e., molecules that are produced by living organisms) or compounds of similar structure, and thus it is appropriate to mention a few generalizations. Proteins are usually thermally unstable and thus have a limited shelf-life. Proteins contain several

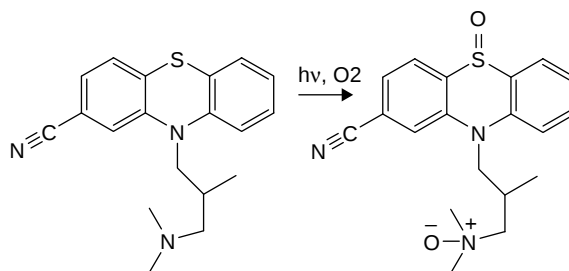


Figure 33 Photooxidation of cyamemazine in O_2 saturated solution.

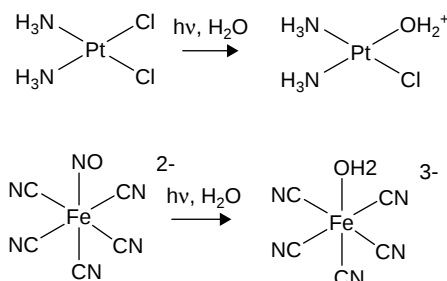


Figure 34 Inorganic compounds photoreaction.

photoreactive sites (S–H, benzylic H, H α to the amide function, electron-rich moieties such as indole or phenol rings) (89). However, photolability gives only a minor contribution to the decomposition of a pure protein exposed to ambient light, since the light absorption of proteins involves UV-C arriving borderline with UV-B and these regions of the spectrum that are scarcely present in the solar radiation. However, during the time required for formulation and packaging of the drug preparation, an (accidental) exposure to UV might result; furthermore some impurity may act as a sensitizer. Under these conditions excitation, in particular, involving tryptophan residues or the action by an impurity leads to abstraction of a hydrogen atom or electron and proton transfer and the formation of radicals. These add dissolved oxygen forming peroxy radicals that in turn abstracts hydrogen pursuing the decomposition and forming unstable hydroperoxides (90,91). The end result is the oxidation of the labile functional groups (sulfide is oxidized to sulfoxide in methionine, to sulfone and sulfonic acids in cysteine; tryptophan undergoes oxidative cleavage of the indole ring and yields kynurenine and its ring-hydroxylated derivatives), as well as the aggregation of the protein due to radical cross-linking. Once initiated, the phenomenon occurs at an increasing rate because some of the products (e.g., kynurenines from tryptophan) absorb light at a longer wavelength than the starting protein and function as effective sensitizers.

Dependence on the Physical State and of Medium Characteristics

Different forms of a molecule, such as tautomers or different ionic states, generally have a different absorption spectrum and may have a different photochemistry, the latter depending on whether the reaction of the excited state is faster than the establishing of the equilibrium between the various forms. Additives acting as acid, base, or buffer may affect the equilibrium between different ionic forms of a drug principle and thus modify the rate of the reaction or the path followed. The result may be either an increase or a reduction in the photolability. Thus, the rate of photodegradation of doxorubicin, daunorubicin, and epirubicin increases in basic

solutions (92), while furosemide is almost photostable in basic solutions but reactive under acidic conditions (93) and nifedipine is decomposed at a rate independent of pH in the range of 2–12 (94). With lomefloxacin, both the rate of degradation and the product distribution change with the pH (95). In evaluating these data, it should be taken into account that equilibria in the excited state may occur at a different pH with respect to that of the ground state. Note also that some of the salts used as buffers may interfere with the photochemistry in other ways. In particular, (hetero)aromatic molecules are often readily reduced. This occurs, e.g., with sulfites, which modify the photochemistry that occurs (96,97). However, a change in the photobehavior may occur also with nonreducing salts, as found, e.g., in the effect of phosphates on the reactivity of some fluoroquinolones (98).

The physical state is obviously important. Mechanistic studies usually involve the irradiation of dilute solutions of the molecule under consideration, in order to ensure a uniform excitation of the solute within the vessel. In this case, the reaction course is cleaner (the further reaction of primary photoproducts and often also side processes are minimized) and a simple kinetic behavior is followed (see above). On the other hand, a much more complex behavior is followed when a concentrated solution is used, where a large (but λ -dependent) fraction of the light is absorbed in the layer close to the lamp. If the compound considered has a high molar absorptivity (and thus the solution has a high optical density) at the wavelength(s) employed, absorption is limited to a thin layer at the surface close to the lamp. As an example, for a 0.1 M solution of a drug with a molar absorptivity $\epsilon = 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, 90% of the light is absorbed in the first 0.1 mm of solution and 99% in 1 mm. Under such a condition, and particularly if the solution is not adequately stirred, multiple photoreactions leading to secondary products or to further degradation occur. Furthermore, a radical-initiated polymerization leading to the formation of a poorly transmitting film on the surface of the vessel often intervenes and under this condition, the bulk of the solution may be scarcely affected even after prolonged irradiation, thus limiting the advancement of the photoreaction.

This issue is even more complex when a photochemical reaction is carried out in the solid state, because of the filter effect by the substrate itself. This inhibits excitation of all but the outmost layers, unless a wavelength window can be chosen that is absorbed by the reagent but not by the photoproduct. In the first case, the external aspect of the crystals may deeply change, which certainly is no improvement of the appearance of the drug preparation, but the therapeutic efficacy unreduced because the extent of decomposition is negligible (99–101). In the second case, layer after layer is converted and a whole crystal is transformed. This is actually observed with some dihydropyridine drugs such as nifedipine, which absorb in the visible light where the photoproduct is transparent. Since the solid state is common in formulation, e.g., in tablets, many studies have been carried out under such conditions. In several cases, a dependence on the form and size of crystals has been revealed (since this can modify the ratio refraction/absorption) (102,103). A different case is when the position of atoms in the crystal lattice directs the photochemical reaction (assumed to involve only moieties sufficiently close one to another. Several examples have been reported for cycloaddition processes, as is the case with the cinnamic acid derivative below. One of the polymorph forms has the C=C bond of pairs of molecules prearranged in the favorable way and undergoes [2+2] cycloaddition, while in the case of the other one, where the C=C bonds are too remote one from another, irradiation leads to polymeric products (Fig. 35) (104).

PHOTOCHEMISTRY OF DRUG PREPARATIONS

Photochemistry of the Excipient

The photolability of a drug preparation obviously presents a much more complex problem than a pure drug substance. Thus, it has to be determined whether the excipients are themselves photolabile, or in which way the drug–excipient interaction (or drug–drug for multicomponent medicines) affects the photochemistry of the drug considered. This issue gains an increasing interest with the development of controlled release pharmaceutical forms. In fact, it

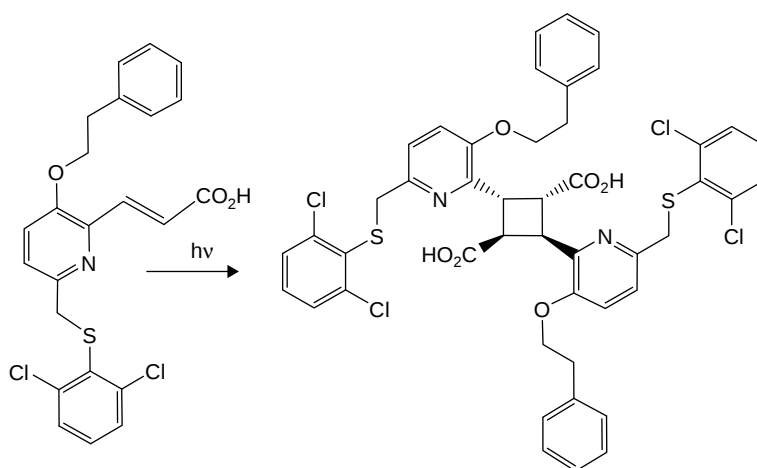


Figure 35 Photocycloaddition with a cinnamic acid derivative in the solid state.

has been demonstrated that the efficiency of the functional polymers used for extended-release formulations is affected by irradiation. As a result, the drug release profile changes proportionally to the exposure time, although the effect depends on the structure of the polymer used. In a study, it has been found that polyethylene oxide is more affected by irradiation than hydroxypropylmethyl cellulose and polyvinyl alcohol, which are quite photostable (105,106). This presumably depends on the hydrogen donating properties of the polymer as well as on the nature of the interaction with the drug. Any factor promoting hydrogen exchange and thus oxidative degradation of the functional polymer via peroxy radicals will facilitate the alteration of the release profile.

Drug–Excipients Interactions

The formulating agents added for various aims may exert a role in the photochemistry of the active principle, either slowing it down or accelerating it. Thus, it is generally advisable that the photolability of potential formulations is assessed, preferably at the preformulation stage. Apart from the photochemistry of the excipient itself considered above, anything different from the drug substance (D), in a general sense an excipient (E), may affect the photochemistry occurring in the drug preparation (reaction i, Fig. 36), as may impurities and dissolved oxygen. As pointed out by Kristensen (107), all of these possibilities should be taken into account. Such excipient may act (ii) as filters, preventing the absorption of light by the drug principle (E absorbs competitively with D); (iii) as sensitizer, transferring electronic energy to a substrate; in this way the excited state of the drug is reached indirectly, but possibly in a more efficient way, e.g., it may lead to the triplet state of a drug that is transparent to the wavelength used; (iv) as quenchers, providing a sink for the electronic energy initially absorbed; (v) as ground state reagents, reacting with the excited state or, more commonly, with a primary intermediate – that in generally will be longer lived; (vi), (vii) as a excited state reagent, consuming D either via its excited state or via a photogenerated reagent R, when excitation of the system generates an aggressive intermediate and thus introduces a new reaction path.

Traces of heavy metal cations, as an example, may affect a photoreaction, and particularly the ubiquitous radical oxidative decomposition, in at least two ways. The first one is quenching of the process from the start by oxidizing an intermediate alkyl radical to a cation (path i, Fig. 37). This prevents the subsequent reaction with oxygen and the peroxy radical-led radical chain degradation. On the other hand, metal ions may activate hydroperoxy groups formed by oxygen addition to the alkyl radical and generate hydroxyl or alkoxy radicals, much more

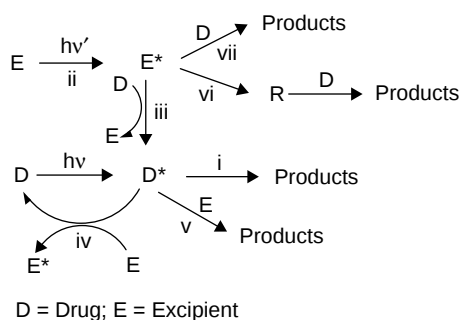


Figure 36 Effect of the excipients on the photochemistry of the drug preparations.

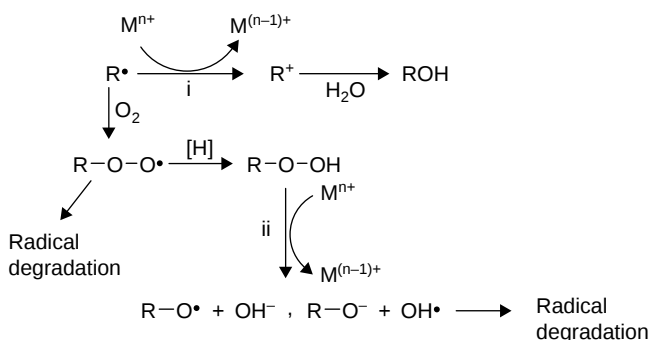


Figure 37 Radical oxidative decomposition mediated by heavy metal cations.

aggressive than peroxy radicals (photo-Fenton reaction, path ii, Fig. 37). This increases the efficiency of hydrogen abstraction from the ground state substrate, facilitating the degradation. The last one appears to be the mechanism behind the observed degradation of estrone in aqueous solution (108).

Chelating agents may prevent the metal-induced effects by complexing the ions, resulting, in a positive or negative effect on the photostability depending on the balance between the above reactions. As an example, with nitrofurazone the rate of degradation decreases in the presence of iron ions, but increases with copper ions (109). The addition of EDTA may increase the photostability of riboflavine (110). On the other hand, the rate of reaction may increase when chelation leads to an increased amount of reactive species (111).

Additives that absorb light competitively prevent excitation of the substrate. UV filters, similar to those used in sunscreens and dyes can be used for photoprotection (and their photostability under applicative conditions has to be tested) (112). However, a strongly absorbing, photostable drug can itself act as a filter and prevent excitation (and thus photoreaction) of the excipient or of another photolabile drug. This appears to be the case for the photostable drug triamterene that protects furosemide both in solution and in tablets (a lucky circumstance since this is a therapeutically effective association) (113).

In this group, one may consider titanium dioxide, a widely used excipient that is meant as a light-protecting agent. In fact, this material reflects a large fraction of the light impinging and thus acts as "physical" filter. However, part of the light is absorbed and this causes transient charge separation on the oxide surface. Although the phenomenon is short-lived, species adsorbed may intercept the charges. Typically, water is oxidized to hydroxyl radicals and oxygen reduced to superoxide anion. The absorption spectrum of this material extends up to 390–400 nm

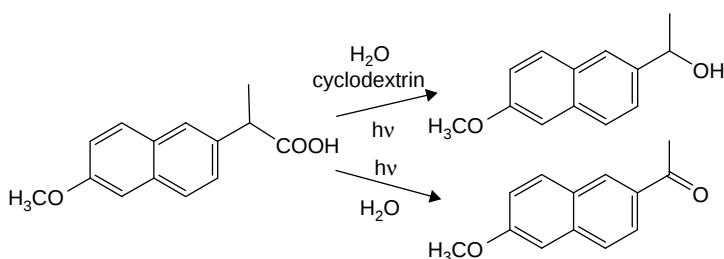


Figure 38 Photodecarboxylation of naproxen: effect of cyclodextrin.

and the OH radicals react with many organic molecules. Therefore, exposure to solar or artificial light may result in a fast degradation of an adsorbed drug. This holds also when the compound considered is transparent to the radiation used, as is the case with epinephrine, a drug stable to ambient light that is photodegraded, however, in a capsule formulation containing TiO_2 (114). A related photodegradation has been observed with several drugs, e.g., with nisoldipine and famotidine. The efficiency of this photocatalyzed degradation is strongly dependent on the allotropes of the oxide (of the crystal forms, anatase is a more efficient photo-oxidant than rutile), to the specific surface area and the relative humidity (115,116).

Drug Complex Formation

Encapsulation in cyclodextrins (CDs) is increasingly applied in formulations because of the greater stability (including photostability) of the drug substance obtained under these conditions (117). As an example, CD complex formation alters tautomeric equilibria, as demonstrated in the case of pyroxicam, and consequently the photochemical properties (118), as well as acid-base equilibria, as in the case of phenylpropionic acids (119). As a matter of fact, complex formation may both enhance and decrease the rate of photoreaction, e.g., both cases have been reported with different arylpropionic acids. The product distribution can likewise change, since a CD may well become involved in the reaction, most often as a hydrogen donor. With naproxen, as an example, photodecarboxylation leads to a mixture of the alcohol and the ketone. The first one is the main product (and is formed in a fast reaction) in CDs, while the latter predominates in neat water (120) (Fig. 38). Liposomes and lipospheres generally reduce the photolability of drugs, as demonstrated, i.e., with dihydropyridines, melatonin, and tretinoin (121–123).

SUMMARY

In summary, the knowledge about the photochemistry of drugs has considerably increased over the last two decades. The ICH guidelines certainly need improvement, but their introduction has fostered attention to the condition of the experiment. If this trend continues, it will become easier to compare data from different laboratories. On the negative side, the number of papers devoted to this topic has not increased. In view of the variety of photochemical paths available to the diverse structures present in drugs, one may wish that more examples are investigated so that generalizations are extended and strengthened. This will help in the safe handling of drugs.

REFERENCES

1. ICH Stability Testing. Photostability Testing of New Drug Substances and Products ICH Harmonized Tripartite Guideline. Geneva: ICH, 1997. Reprinted in ref. 9, pp. 66–73.
2. Albini A, Fasani E. Drugs: Photochemistry and Photostability. Cambridge: The Royal Society of Chemistry, 1998: 66–73.

3. Federal Register, 1997; 62: 27115–22.
4. ICH: Draft Guidance for Industry: Stability Testing of Drug Substances and Drug Products. 1998.
5. Pharmacopeial previews. Drugs and excipients – USP general information chapters. Pharmacop Forum 2000; 26: 384–8.
6. Thatcher SR, Mansfield RK, Miller RB, Davis CW, Baertschi SW. Pharmaceutical photostability: a technical guide and practical interpretation of the ICH Guideline and its application to pharmaceutical stability. *Pharmaceut Technol Part II* 2001; 25(3): 98–110.
7. Thatcher SR, Mansfield RK, Miller RB, Davis CW, Baertschi SW. Pharmaceutical photostability: a technical guide and practical interpretation of the ICH Guideline and its application to pharmaceutical stability. *Pharm Technol Part II* 2001; 25(4): 50–62.
8. Aman W, Thoma K. ICH guidelines for photostability testing. Aspects and directions of use. *Pharmazie* 2003; 58: 877–80.
9. Albini A, Fasani E. *Drugs: Photochemistry and Photostability*. Cambridge: The Royal Society of Chemistry, 1998.
10. Sager N, Baum R, Wolters R, Layloff T. Photostability testing of pharmaceutical products. *Pharmacop Forum* 1998; 24: 6331–3.
11. Tønnesen H, Karlsen J. A comment on photostability testing according to ICH Guideline: calibration of photolysis sources. *J Pharmeuropa* 1997; 9: 735–6.
12. Helboe P. The elaboration and application of the ICH Guideline on photostability: a European prospective. In ref 9, pp. 243–6.
13. Riehl J, Maupin C, Layloff T. On the choice of a photolysis source for the photostability testing of pharmaceuticals. *Pharmacopeial Forum* 1995; 21: 1654–63.
14. Piechocki J. Selecting the right source for pharmaceutical photostability testing. In ref. 9, pp. 247–71.
15. Anderson NH. Photostability testing design and interpretation of tests on drug substances and dosage forms. In ref. 23, pp. 305–22.
16. Sequeira F, Vozzone C. Photostability studies of drug substances and products. Practical implications of the ICH guideline. *Pharm Tech* 2000; 24(7): 30–5.
17. Baertschi SW, Alsante KM, Tonnesen HH. Commentary: a critical assessment of the ICH guideline on photostability testing of new drug substances and products (Q1B). *J Pharm Sci* 2010; 99: 2934–40.
18. Bonneau R, Jousset-Dubien J. A trans cyclohexene. *J Am Chem Soc* 1976; 98: 4329–30.
19. Kropp PJ. Photochemistry of cycloalkenes. III. Ionic behaviour in protic media and isomerization in aromatic hydrocarbon media. *J Am Chem Soc* 1967; 89: 5199–208.
20. Cohen SG, Sherman W. Inhibition and quenching of the light induced reduction of benzophenone to benzopinacol and to benzhydrol. *J Am Chem Soc* 1963; 85: 1642–7.
21. Horspool WH, Song PS (eds). *CRC Handbook of Organic Photochemistry and Photobiology*, Boca Raton: CRC Press, 1995.
22. Horspool WH, Armesto D. *Organic Photochemistry: A Comprehensive Treatment*, New York: Ellis Horwood, 1992.
23. Turro NJ. *Modern Molecular Photochemistry*. Menlo Park: Benjamin Cummings, 1978.
24. Tønnesen HH. *Photostability of Drugs and Drug Formulation*. Boca Raton: CRC Press, 1996.
25. Fasani E, Mella M, Monti S, Sortino S, Albini A. The photochemistry of metyrapone. *J Chem Soc Perkin Trans 2* 1996; 1819–22.
26. Ricci A, Fasani E, Mella M, Albini A. General patterns in the photochemistry of pregna-1, 4-dien-3, 20- diones. *J Org Chem* 2003; 68: 4361–6.
27. Iqbal J, Gupta A, Husain A. Photochemistry of clobetasol propionate, a steroidal anti-inflammatory drug. *ARKIVOC* 2006; 11: 91–8.
28. Takács M, Ekiz Gücer N, Reisch J, Gergely-Zobin A. Light-sensitivity of corticosteroids in the crystalline state. *Pharm Acta Helv* 1991; 66: 137–40.
29. Natarajan A, Tsai C K, Khan Saeed I, McCarran P, Houk K N, Garcia-Garibay M A. The photoarrangement of α -santonin is a single-crystal-to-single-crystal reaction: a long kept secret in solid-state organic chemistry revealed. *J Am Chem Soc* 2007; 129: 9846–7.
30. Fasani E, Dondi D, Ricci A, Albini A. Photochemistry of 4-(2-Nitrophenyl)-1,4-dihydropyridines. Evidence for electron transfer and formation of an intermediate. *Photochem Photobiol* 2006; 82: 225–30.
31. Moore DE, Wilkins BJ. Common products from gamma radiolysis and ultraviolet photolysis of metronidazole. *Radiat Phys Chem* 1990; 36: 547–50.
32. Frith RG, Phillipou J. Application of clomiphen photolysis to assay based on analysis of the derived phenanthrenes. *J Chromatogr* 1986; 367: 260–66.

33. Allwood MC, Plane JH. The wavelength-dependent degradation of vitamin A exposed to ultraviolet radiation. *Int J Pharm* 1986; 31: 1–7.
34. Montanaro S, Lhiaubet-Vallet V, Iesce MI, Previtera L, Miranda MA. A mechanistic study on the phototoxicity of atorvastatin: singlet oxygen generation by a phenanthrene-like photoproduct. *Chem Res Toxicol*. 2009; 22: 173–8.
35. Mermet-Bouvier R. Photochemistry of Vitamin D₂. *Bull Soc Chim Fr* 1973; 11: 3023–6.
36. Snoeren AEC, Daha MR, Lugtenburg J, Havinga E. Studies of Vitamin D and related compounds. Part 21. Photosensitized reactions. *Rev Trav Chim Pays Bas* 1970; 89: 261.
37. Fabre H, Ibork H, Lerner DA. Photodegradation kinetics under UV light of aztreonam solutions. *J Pharm Biomed Anal* 1992; 10: 645–50.
38. Iqbal J, Gupta A, Husain A. Photochemistry of phenazopyridine hydrochloride. *Pharmazie*. 2006; 61: 747–50.
39. Lerner DA, Bannefond G, Fabre H, Mandrou B, Simeon de Buochberg M. Photodegradation paths of cefatoxim. *J Pharm Sci* 1988; 77: 699–703.
40. Quillian MA, McCurry BE, Hoo KH, McCalla DR, Weitekunas S. Identification of the photolysis products of nitrofurazone irradiated under laboratory illumination. *Can J Chem* 1987; 65: 1128–32.
41. Piñero LE, García C, Lhiaubet-Vallet V, Oyola R, Miranda MA. Photophysics and photochemistry of Z-chlorprothixene in acetonitrile. *Photochem Photobiol*. 2000; 85: 895–900.
42. Moore DE, Roberts-Thompson S, Zhen D, Duke CC. Photochemical studies on the anti-inflammatory drug diclofenac. *Photochem Photobiol* 1990; 52: 685–90.
43. Paillous N, Verrier M. Photolysis of amiodarone, an antiarrhythmic drug. *Photochem Photobiol* 1988; 47: 337–43.
44. Fasani E, Barberis Negra FF, Mella M, Monti S, Albini A. Photoinduced C–F bond cleavage in some fluorinated 7-amino-4-quinolone-3-carboxylic acids. *J Org Chem* 1999; 64: 5388–95.
45. Freccero M, Fasani E, Mella M, Manet I, Monti S, Albini A. Modelling the photochemistry of the reference phototoxic drug lomefloxacin, by experiments and DFT and post-HF methods. *Chemistry A- Eur. J*. 2008; 14: 653–63.
46. Fasani E, Tilocca F, Albini A. Photochemistry of oxazolidinone antibacterial drugs. *Photochem Photobiol* 2009; 85: 879–85.
47. Boreen AL, Arnold W, McNeill A. Photochemical fate of sulfa drugs in the aquatic environment: sulfa drugs containing five-membered heterocyclic groups. *Environ Sci Technol* 2004; 38: 3933–40.
48. Shih K. Photodegradation products of chloramphenicol in aqueous solution. *J Pharm Sci* 1971; 60: 1889–90.
49. Chahidi C, Giraud M, Aubailly M, Valla A, Santus R. 2,4-Diamino-6-pteridinecarboxyaldehyde and an azobenzene derivative are produced by UV photodegradation of methotrexate. *Photochem Photobiol* 1986; 44: 231–3.
50. Vargass F, Rivas C, Miranda MA, Bosca F. Photochemistry of the non-steroidal anti-inflammatory drugs, propionic acid derived. *Pharmazie* 1991; 46: 767–71.
51. Encinas S, Miranda MA, Marconi G, Monti S. Triplet photoreactivity of the diaryl ketone tiaprofenic acid and its decarboxylated photoproduct. *Photobiological implications*. *Photochem Photobiol* 1998; 67: 420–5.
52. Borsarelli CD, Braslavsky SE, Sortino S, Marconi G, Monti S. Photodecarboxylation of ketoprofen in aqueous solution. A timeresolved laser-induced optoacoustic study. *Photochem Photobiol* 2000; 72: 163–71.
53. Jiménez MC, Miranda MA, Tormos R, Vayá I. Characterisation of the lowest singlet and triplet excited states of S-flurbiprofen. *Photochem Photobiol Sci* 2004; 3: 1038–41.
54. Cosa G. Photodegradation and photosensitization in pharmaceutical products: assessing drug phototoxicity. *Pure Appl Chem* 2004; 76: 263–75.
55. Musa Klefah AK, Eriksson LA. Photodegradation mechanism of nonsteroidal anti-inflammatory drugs containing thiophene moieties: suprofen and tiaprofenic acid. *J Phys Chem B* 2009; 113: 11306–13.
56. Uwai K, Tani M, Ohtake Y, Abe S, Maruko A, Chiba T, Hamaya Y, Ohkubo Y, Takeshita M. Photodegradation products of propranolol: the structures and pharmacological studies. *Life Sci*. 2005; 78: 357–65.
57. Castell JV, Gomez Lechon MJ, Mirabet V, Miranda MA, Morea IM. Photolytic degradation of benorylate: effects of the photoproducts on cultured hepatocytes. *J Pharm Sci* 1987; 76: 374–8.
58. Reisch J, Ekiz-Gücer N, Takacs M, Gunaherath GM, Kamal B. The photoisomerization of azapropazone. *Arch Pharm (Weinheim)* 1989; 322: 295–6.

59. Albini A, Alpegiani M, Borghi D, Del Nero S, Fasani E, Perrone E. Solid state photoreactivity of a dioxolonenomethyl ester. *Tetrahedron Lett* 1995; 36: 4633–6.
60. Fabre H, Hussam-Eddine N, Lerner D, Mandrou B. Stability-indicating assay for phenylbutazone: high-performance liquid chromatographic determination of hydrazobenzene and azobenzene in degraded aqueous phenylbutazone solutions. *J Pharm Sci* 1984; 73: 1706–9.
61. Marciniak B. Photochemical decomposition of phenazone derivatives: part 3. Kinetics of photolysis in aqueous solutions. *Pharmazie* 1984; 39: 103–6.
62. Reisch AF. Photo and radiochemical studies: V. Decomposition of aqueous amidopyrine solutions under the influence of light and γ -rays. *Deut Apoth-Ztg* 1967; 107: 1358–159.
63. Chiang HC, Li SY. Photochemistry of 5,5-diphenyltoin. *J Taiwan Pharm Assoc* 1977; 29: 70–6.
64. Andersin R, Ovaskainen J, Kaltia S. Photochemical decomposition of midazolam: III. Isolation and identification of products in aqueous solutions. *J Pharm Biomed Anal* 1994; 12: 165–72.
65. Nudelman NS, Cabrera CG. Isolation and structural elucidation of degradation products of alprazolam: photostability studies of alprazolam tablets. *J Pharm Sci* 2002; 91: 1274–86.
66. Cornelissen PJG, Beijersbergen van Henegouwen GMJ, Gerritsma KW. Photochemical decomposition of 1,4-benzodiazepines. Chlordiazepoxide. *Int J Pharm* 1979; 3: 205–20.
67. Roth HJ, Adomeit M. Photochemistry of nitrazepan. *Arch. Pharm. (Weinheim)* 1973; 306: 889–97.
68. Reisch J, Ekiz-Gücer N, Tewes J. Photochemical studies: LXIII. Photostability of some 1,4-benzodiazepines in the crystalline state. *Liebigs Ann Chem* 1992; 69–70.
69. Barton H, Bojarski J, Mokrosz J. Photochemical ring opening of barbital. *Tetrahedron Lett.* 1982; 23: 2133–4.
70. Barton HJ, Bojarski J, Zurowska A. Stereospecificity of the photoinduced conversion of methylphenobarbital to mephentoin. *Arch. Pharm. (Weinheim)* 1986; 319: 457–61.
71. Barton HJ, Mokrosz J, Bojarski J, Klimczak M. Products of photolysis and hydrolysis of pentobarbital. *Pharmazie* 1980; 35: 155–8.
72. Ekiz-Gücer N, Reisch J. Photostability of minoxidil in the liquid and in the solid state. *Acta Pharm Turc* 1990; 32: 103–6.
73. Cermola F, Della Greca M, Iesce M R, Previtera L, Rubino M, Temussi F. Photoreactivity of triazolopyridinones, including the drug trazodone, in aqueous solution. *J Photochem Photobiol A: Chemistry* 2009; 206: 198–204.
74. Foote CF. Definition of type I and type II photosensitized oxidation. *Photochem Photobiol* 1991; 54: 659.
75. Tønnesen HH, Grislingaas AL, Woo SO, Karlsen J. Photochemical stability of antimalarials: I. Hydroxychloroquine. *Int J Pharm* 1988; 43: 215–19.
76. Iqbal J, Husain A, Gupta A. Photooxidation of acyclovir in aqueous solution. *Pharmazie* 2005; 60: 574–6.
77. Andrisano V, Bertucci C, Battaglia A, Cavrini V. Photostability of drugs: photodegradation of melatonin and its determination in commercial formulations. *J Pharm Biomed Anal* 2000; 23: 15–23.
78. De Mol NJ, Beijersbergen van Henegouwen GMJ, Gerritsma KW. Photochemical decomposition of catecholamines: II. The extent of aminochrome formation from adrenaline, isoprenaline and noradrenaline under ultraviolet light. *Photochem Photobiol* 1979; 29: 479–82.
79. Sedee AGJ, Beijersbergen van Henegouwen GMJ. Photochemical decomposition of contraceptive steroids: a possible explanation for the observed (photo)allergy of the oral contraceptive pill. *Arch Pharm (Weinheim)* 1985; 318: 111–19.
80. Dedola G, Fasani E, Albini A. The photoreactions of trimethoprim in solution. *J Photochem Photobiol A* 1999; 123: 47–51.
81. Lupon P, Grau F, Bonet JJ. The photooxygenation of $\Delta^{9(11)}$ -dehydroestrone and its 3-methyl ether. *Helv Chim Acta* 1984; 67: 332–3.
82. Vargas F, Carbonell K, Camacho M. Photochemistry and in vitro phototoxicity studies of levomepromazine (methotrimeprazine), a phototoxic neuroleptic drug. *Pharmazie* 2003; 58: 315–19.
83. Tammilehto S, Torniainen K. Photostability of dothiepin in aqueous solution. *Int J Pharm* 1989; 52: 123–8.
84. Glass BD, Brown ME, Drummond PM. Photoreactivity *versus* activity of a selected class of phenothiazines: a comparative study. In ref. 9, pp. 134–49.
85. Bosà F, Morlière P, Miranda MA, Castell J, Santus R. Primary steps of the photochemical reactions of 2-cyano-10-(3-[dimethylamino,*N*-oxide]-5-oxide-phenothiazine), the photoproduct of cymemazine, a phototoxic neuroleptic: comparison with the sulfoxide. *Photochem Photobiol Sci* 2006; 5: 336–42.

86. Davidson SW, Lyall D. Sodium nitroprusside stability in light-protected administration sets. *Pharm J* 1987; 239: 599–600.
87. Macka M, Borak J, Semenkova L, Kiss F. Decomposition of cisplatin in aqueous solutions containing chlorides by ultrasonic energy and light. *J Pharm Sci* 1994; 83: 815–18.
88. Moucheron C. From cisplatin to photoreactive Ru complexes: targeting DNA for biomedical applications. *N J Chem* 2009; 33: 235–45.
89. Kerwin BA, Remmelle RL. Protect from light: photodegradation of protein biologics. *J Pharm Sci* 2007; 96(6), 1468–79.
90. Rathore N, Rajan SR. Current perspectives on stability of protein drug products during formulation, fill and finish operations. *Biotechnol Prog* 2008; 24: 504–14.
91. Sobolewski AL, Domcke W. Relevance of electron-driven proton-transfer processes for the photostability of proteins. *Chem Phys Chem* 2006; 7: 561–4.
92. Wood MJ, Irwin WJ, Scott DK. Photodegradation of doxorubicin, daunorubicin and epirubicin measured by high-performance liquid chromatography. *J Clin Pharm Therap* 1990; 15: 291–300.
93. Bundgaard H, Nørgaard T, Nielson NM. Photodegradation and hydrolysis of furosemide and furosemide ester in aqueous solution. *Int J Pharm* 1988; 42: 217–24.
94. Thoma K, Klimek R. Investigations of photoinstability of nifedipine: part 2. Influence of medium conditions. *Pharm Ind* 1985; 47: 319–26.
95. Fasani E, Mella M, Albini A. Photochemistry of the phototoxic drug lomefloxacin: paths observed in the presence of amines or NaOH and from the methyl ester. *Eur J Org Chem* 2004; 5075–82.
96. Brustugun J, Kristensen S, Tønnesen HH. Photostability of symphatomimetic agents in commonly used infusion media in the absence and presence of bisulfite. *PDA J Pharm Sci Technol* 2004; 58: 296–308.
97. Brustugun J, Kristensen S, Tønnesen HH. Photostability of epinephrine – influence of bisulfite and degradation products. *Pharmazie* 2004; 59: 457–63.
98. Fasani E, Mella M, Monti S, Albini A. Unexpected photoreaction of some 6-fluoro-7-aminoquinolones in phosphate buffer. *Eur J Org Chem* 2001; 391–7.
99. Merrifield R, Carter PL, Clapham D, Sanderson FD. Addressing the problem of light instability during formulation development. In ref. 23, pp. 141–54.
100. Nyqvist H. Light stability testing of tablets in the Xenotest and the Fadeometer. *Acta Pharm Suec* 1984; 21: 245–52.
101. Matsuda Y, Masahara R. Comparative evaluation of coloration of photosensitive solid drugs under various light sources. *Yakagaku Zasshi* 1980; 100: 953–7.
102. Kawabe Y, Nakamura H, Hino E, Suzuki S. Photochemical stability of some dihydropyridine calcium-channel blockers in powdered pharmaceutical tablets. *J Pharm Biomed Anal* 2008; 47: 618–24.
103. Fiori J, Gotti R, Bertucci C, Cavrini V. Investigation on the photochemical stability of lercanidipine and its determination in tablets by HPLC-UV and LC-ESI-MS/MS. *J Pharm Biomed Anal* 2006; 41: 176–81.
104. Orford c, Webb ML, Cattanach KH, Cottee FH, Escott RE, Pitfield ID, Richards JJ. An analytical and structural study of the photostability of some leukotriene B₄ antagonists. In: Albini A, Fasani E, eds. *Drugs: Photochemistry and Photostability*. Cambridge: The Royal Society of Chemistry, 1998; 182–93.
105. Maggi L, Ochoa Machiste E, Fasani E, Albini A, Segale L, Conte U. Photostability of extended release matrix formulations. *Eur J Pharm Biopharm* 2003; 55: 99–105.
106. Ochoa Machiste E, Segale L, Conti S, Fasani E, Albini A, Conte U, Maggi L. Effect of UV light exposure on hydrophilic polymers used as drug release modulators in solid dosage forms. *J Drug Del Sci Tech* 2005; 15: 151–7.
107. Kristensen S. Photostability in parenteral products. In: Tønnesen HH, ed. *Photostability of Drugs and Drug Formulations*, 2nd edn. Boca Raton, FL: CRC Press, 2004; 303–30.
108. Feng X, Ding S, Tu J, Wu F, Deng N. Degradation of estrone in aqueous solution by photo-Fenton system. *Sci Total Environ* 2005; 345: 229–37.
109. Shahjahan M, Enever RP. Photolability of nitrofurazone in aqueous solution: II. Kinetic studies *Int J Pharm* 1996; 143: 83–92.
110. Asker AF, Habib MJ. Effect of certain stabilizers on photobleaching of riboflavin solutions. *Drug Dev Ind Pharm* 1990; 16: 149–56.
111. Halliwell B, Gutteridge JMC. *Free Radicals in Biology and Medicine*. Oxford: Clarendon Press, 1985; 18, 26, 52, 124.

112. Piecocki JT, Thoma K. *Pharmaceutical Photostability and Stabilization Technology*. Boca Raton: CRC Press, 2006.
113. Fiori J, Ballardini R, Andrisano V, Cavrini V. Photostability studies on the furosemide-triamterene drug association: *Il Farmaco* 2003; 58: 867–73.
114. Templeton AC, Bowen WE, Klein LJ, Harmon PA, Lu Y, Reed RA. Unexpected photochemistry in pharmaceutical products: a review on the role of diluents, excipients, and product components in promoting pharmaceutical photochemistry. *Drugs Pharm Sci* 2007; 163: 223–51.
115. Kakinoki K, Yamane K, Igarashi M, Yamamoto M, Teraoka R, Matsuda Y. Evaluation of titanium dioxide as pharmaceutical excipient for preformulation of a photo-labile drug: effect of physicochemical properties on the photostability of solid-state nisoldipine. *Chem Pharm Bull* 2005; 53: 811–15.
116. Kakinoki K, Yamane K, Teraoka R, Otsuka M, Matsuda Y. Effect of relative humidity on the photocatalytic activity of titanium dioxide and photostability of famotidine, *J Pharm Sci* 2004; 93: 582–9.
117. El-Kemary M, Douhal A. In Douhal A., ed. *Cyclodextrine Materials: Photophysics, Photochemistry, Photobiology*. Amsterdam: Elsevier, 2006; Chapter 4.
118. Xiliang G, Yu Y, Guoyan Z, Guomei Z, Jianbin C, Shaomin S. Study on inclusion interaction of piroxicam with B-cyclodextrin derivatives. *Spectrochim Acta Part A* 2003; 3379–86.
119. Monti S, Sortino S. Photoprocesses of photosensitizing drugs within cyclodextrin cavities. *Chem Soc Rev* 2002; 31: 287–300.
120. Jimenez MC, Miranda MA, Tormos R. Photochemistry of naproxen in the presence of β -cyclodextrin. *J Photoche Photobiol A: Chem* 1997; 104: 119–21.
121. Ragno G, Risoli A, Ioele G, Cione E, De Luca M. Photostabilization of 1,4-dihydropyridine antihypertensives by incorporation into β -cyclodextrin and liposomes. *J Nanosci Nanotechnol* 2006; 6: 2979–85.
122. Tursilli R, Casolari R, Iannuccelli V, Scalia S. Enhancement of melatonin photostability by encapsulation in lipospheres. *J Pharm Biomed Anal* 2006; 40: 910–14.
123. Ioele G, Cione E, Risoli A, Genchi G, Ragno G. Accelerated photostability study of tretinoin and isotretinoin in liposome formulations. *Int J Pharm* 2005; 293: 251–60.

8 | Practical aspects of conducting photostability stress testing

David Clapham, Allen C. Templeton, Lee J. Klein, and Mark H. Kleinman

INTRODUCTION

The photostability of active pharmaceutical ingredients (API) and drug products (DP) may impact shelf life, handling, packaging, and even photosafety of the product (e.g., phototoxicity and photogenotoxicity). Hence, photostress testing is an important component of the drug development process and it is important to understand the intrinsic photoreactivity of the API early in development. A photo-unstable API may be

- problematic to isolate;
- difficult to formulate under typical conditions in a DP manufacturing suite;
- complex to pack for shipping to the clinic/pharmacy;
- difficult to dose (e.g., if it is administered topically or by infusion);
- difficult to analyze if unstable in a typically lit laboratory (vials may need to be wrapped in aluminum foil or otherwise protected from light);
- an indicator for photosafety concerns that *may* require further investigation.

Thus, a photo-unstable API or product may involve higher development and commercialization costs. A number of books (1–4) provide excellent reference to the topic of photostability of drugs. This chapter focuses on the practical aspects of conducting photostability studies in the context of stress testing (forced degradation). In this chapter, you will find discussion of key topics including

- a discussion and comparison of ICH Option 1 and 2 conditions;
- relevance and rationale for how much light exposure is required;
- advice on sample presentation in order to achieve consistent light exposure;
- how to conduct analytical assessments related to photostability and utilize the data to make critical decisions.

Background

Light Absorption

Light absorption by a molecule, whether drug or excipient, is required for photoreactivity. The UV-Vis absorption spectrum may therefore provide a clue as to the likelihood that a molecule will react. However, light absorption alone does not necessarily lead to photodegradation. What is important is how the molecule can dissipate the energy it absorbs. There are several mechanisms by which absorbed energy can be removed from the molecule without causing bond breakage. Conversely, absorption of only a small number of photons (as indicated by a “tail” in the absorption spectrum that overlaps with the emission spectrum of the light source) may cause degradation of some molecules. Subtle molecular changes can have a marked effect on the ability of a molecule to dissipate energy. Equally, it is possible that although the molecule itself does not appear to absorb any light, the system (such as a formulation) in which it is present does absorb light and can then transfer the absorbed energy to the molecule causing bond breakage and hence degradation.

Thus, it is not possible to directly deduce whether an API is capable of being photoreactive or whether it has the potential to form reactive oxygen species such as singlet oxygen or superoxide from the UV-Vis spectrum alone. Not only is this important in terms of molecular integrity, but there is also a growing literature that clearly demonstrate links between photostability and phototoxicity (5–7) or photogenotoxicity (8). Consequently, one needs to practically assess the photostability of API and drug product in detail before being able to understand the risks and benefits to the patient.

THE ICH Q1B GUIDELINE

Before the issuance of ICH guideline Q1B over 10 years ago (9), a wide variety of practices (different photolytic sources, spectral ranges, exposures, and protocols) were in place (10). The guideline helps to clarify and standardize light radiation sources and the amounts of exposure needed to assess the photostability of drug substances and drug products. However, it does not specifically address the photostability of a product under in-use conditions, the photostability of analytical samples, nor does it delve into the details about the specific requirements from a stress testing (forced degradation) perspective.

ICH Q1B divides the types of photostability studies into confirmatory and “forced degradation” studies. In a confirmatory study, standardized cGMP conditions are used to establish the photostability characteristics of the API and DP. These are akin to the formal long-term or accelerated stability studies as described in the ICH Q1A Stability guideline. Confirmatory studies are usually performed on representative API and drug product meant to reflect the final commercial materials. The studies are primarily designed to assure that the pack is appropriate for the protection of the API and drug product. Forced degradation testing studies are those studies undertaken to purposely degrade the sample. These studies are used to evaluate the overall photosensitivity of the material for method development purposes (specificity), to elucidate degradation pathways and to guide controls needed for the manufacture, packaging, storage, and use of API and drug product. They can also be used to help predict the potential for phototoxicity.

Frequently, since these stress studies are more of a research investigation to help uncover the intrinsic stability of the compound, they may be conducted in a non-GMP manner. Their design should be driven by the application of scientific principles and help to predict the outcome of the confirmatory studies. For molecules that are inherently photo-unstable, the Q1B confirmatory test with its fixed conditions may in fact be harsher than a forced degradation paradigm. This chapter will focus on the photostress experiments (“forced degradation” studies) and will review suggested study designs and potential pitfalls of photolytic stress testing studies.

SPECTRAL CONSIDERATIONS

Typical outdoor and interior light exposure consists of a mixture of UV [UV-B (280–320 nm); UV-A (320–400 nm)] and visible light (400–800 nm) in varying proportions. Outdoors, both ultraviolet and visible light are emitted from the sun. Most of the radiation below 300 nm is filtered out by the ozone layer. Indoor exposure may result from solar radiation that is filtered by window glass (usually with a cut-off transmittance ≥ 315 nm) but the sample may also experience light from additional sources such as fluorescent lights (which may also emit low levels of UV-B radiation). Which part of the spectrum is most destructive to a particular molecule or product is hard to predict. There can be particular “causative wavelengths” where absorption of photons of that wavelength may lead to particularly destructive bond breakage. If this is so, then a light source that is particularly rich in these wavelengths may be more destructive for the molecule than another with nominally more irradiance but with less energy in this part of the spectrum. Thus, lamps with different spectral power distributions can initiate different photodegradation rates and photochemistries.

It is critical to challenge the API and drug product with both UV and Vis radiation because both API and products may be exposed to UV or Visible light during manufacture, shelf life, or even after dosing if the drug product is applied topically or if the API partitions to the skin or eyes. Generally, we recommend that the formal confirmatory photostability studies should be conducted using the same ICH option as the photostress (forced degradation) studies.

The guidance provides two light source options for study as described below. It is important to note that it is possible to obtain different results from each option for certain APIs depending on their UV-Vis absorption profiles, the energy required to initiate the photoinduced reaction (11,12) and the mechanism of photodegradation.

Option 1

Any light source that can produce an output similar to the D65/ID65 emission standard such as an artificial daylight fluorescent lamp combining visible and UV outputs, xenon, or metal halide lamp may be used. These types of radiation sources mimic daylight or indirect filtered light through a glass window to a greater or lesser extent. As such, the radiation sources expose the samples to UVB, UVA, and visible light simultaneously. It is usually appropriate to use an equivalent of the ID₆₅ standard by using a filter because it is unlikely that the API or product will be exposed to direct sunlight for a significant length of time during manufacture, shipping, handling, storage, or use.

Option 1 provides a realistic mimic of sunlight and can therefore be considered to be representative of exposure when sunlight is present (e.g., outdoors or inside a room with a window, etc.). However, if a sample is left in a photochamber with a target of meeting the visible light exposure requirements, then the sample is exposed to an overabundance of UV light relative to the ICH target since sunlight (and those lamps that mimic it) are relatively rich in UV relative to visible light.

Option 2

For ICH Option 2 the same sample is exposed to both a cool white fluorescent and near ultra-violet lamp either simultaneously (all lamps are “on” concurrently) or in series (lamps are “on” in sequence, often in different light chambers). This allows delivery of exact light doses according to the ICH criteria but may not mimic “real world” exposure, although it does simulate indoor lighting conditions when these are composed of glass-filtered daylight and artificial radiation provided by white fluorescent lamps. The cool white fluorescent (visible) lamp is designed to produce an output similar to that specified in ISO 10977(1993) (revised to ISO 18909:2006). The near-UV fluorescent lamp has a spectral distribution from 320 nm to 400 nm with a maximum energy emission between 350 nm and 370 nm. A significant proportion of UV should be in both bands of 320–360 and 360–400 nm. Note however that there is an emission gap between the two sources (380–420 nm) where there is little output from either lamp, so caution should be taken if the API absorbs strongly in this region or if wavelengths in this region cause photo-instability.

As shown in Table 1 (10) below (see also Ref. 13), each option has advantages and disadvantages and either can be used for photostress studies.

Table 1 Advantages and Disadvantages of Option 1 and Option 2

Practical issue	Comment	Rationale
Heat exposure	Option 1 exposes samples to more heat than Option 2	Option 1 lamp sources are more intense and create more heat. Option 1 usually requires a cooling unit which may provide an issue for sample presentation due to blowing of air
Exposure time	Option 1 requires less time for irradiation	Typically, Option 2 is conducted sequentially and with less intense lamps
Spectral gap	Option 2 features multiple lamps which have poor overlap or poor output between 380 and 400 nm	Option 1 has a single lamp with broad spectral distribution
Overexposure	Use of Option 1 typically results in overexposure of UV component	Lamps used for Option 1 (e.g., Xenon) emit a large amount of UV, so it takes longer for the sample to be exposed to the required level of visible light
Spectral range	Option 1 mimics outdoor daylight, Option 2 mimics indoor light	Refer to ISO standards

The choice of which to use will depend on the scientific question being asked. For a full understanding of the photostability behavior of an API or DP, it may be necessary to use both ICH Option light sources. Results from these studies can often be successfully extrapolated to other light sources but there may be value in conducting experiments with light sources that directly match or mimic those actually present in the environment where the sample receives greatest exposure (e.g., manufacturing and packing facility or hospital pharmacy).

OPTION 1 AND OPTION 2: EXPOSURE REQUIREMENTS

Regardless of which option is chosen, in a confirmatory study, samples should be exposed to light providing an overall illumination of not less than 1.2 million lux hours and an integrated near ultraviolet energy of not less than 200 W hours/m². There are no explicit light exposure requirements for photostress studies.

At one extreme, if the compound is highly photolabile, it might be necessary to use much lower light doses than for the confirmatory test in order to ensure that the primary photo products are seen. Generally, this can be achieved with an irradiance that leads to approximately 10–20% overall degradation, preferably with intermediate time points.

On the other hand, what if the compound is photostable? By analogy to the guidance for thermal forced degradation studies (e.g., long-term storage at 30°C compared with accelerated testing at 40°C and stress testing up to 80°C), exposure of a sample to a light dose approximately double that in the confirmatory (formal stability) study (which results in 2.4 million lux hours of visible light and >400 W hours/m² integrated UV energy) should be more than adequate. Keeping the light dose to 2xICH exposure levels is practical as it ensures efficient use of the light stability chamber and reduces the likelihood of secondary photoproducts. If a sample has not shown significant degradation within this period it is unlikely to do so with further exposure. Furthermore, the light exposure for a confirmatory study reflects what is thought to be roughly equivalent to 3 months of continuous exposure to UV and visible light without protective packaging in pharmacy, warehouse or home (11). So, the photostress tests are doubling this “worst-case scenario” and any further extension of the study is, in a practical sense, likely to represent an unrealistic scenario. While there are examples in the literature of the use of up to 10-fold ICH exposure levels for photostress tests, we recommend the use of twofold ICH conditions for standard photostress studies.

In summary, photostress testing studies should employ sufficient light input to achieve an end point of 10–20% degradation or 2xICH exposure if no significant change has occurred.

Practical Considerations

When designing a photostability study, a number of practical issues need to be considered.

Sample Presentation

The major factors that need to be considered in placing samples within a light cabinet are as follows:

- nonuniformity of illumination within the cabinet,
- ensuring that the sample is appropriately exposed to the light source,
- the distance from the light source and hence the dose received,
- container and closure,
- effect of humidity,
- effect of heating.

Nonuniformity of Illumination

All light cabinet manufacturers attempt to obtain uniform illumination within their cabinets using various techniques such as lamp placement, mirrored, or white painted surfaces, etc.

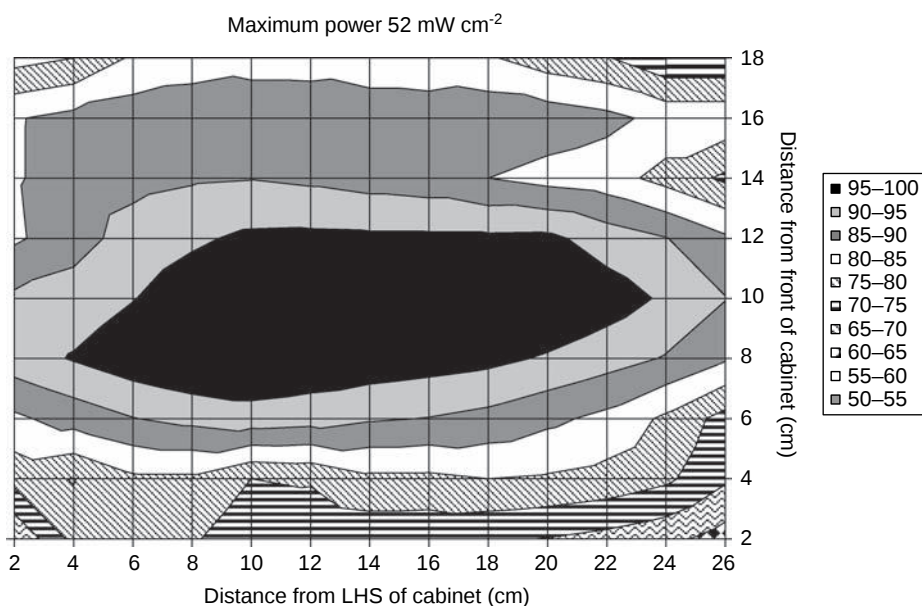


Figure 1 Percentage distribution of power within a xenon arc cabinet (Option 1).

However, illumination can never be completely uniform and all cabinets have “hot spots” and “shaded areas” where the illumination intensity is relatively higher (hot spot) or lower (shaded area). Additionally, if the walls of the cabinet are not entirely clean or of inappropriate construction, it is possible that the spectral power distribution may also change due to selective absorption or reflection of certain wavelengths. This can be mapped using techniques such as actinometry, radiometry, or spectral radiometry.

An example power map is shown in Figure 1.

It is clear that it is important to have a good understanding of the illumination conditions (such as the optical power and ideally the spectral power distribution) at the point in the cabinet where the sample is placed. By extension, care should be taken when comparing results from samples placed at very different locations within a cabinet or in different cabinets. Similarly, sample chambers should not be overfilled; otherwise, differences in results may be due to differential illumination or shading from neighboring samples.

Chamber mapping measurements are not *mandated* by ICH Q1B; however, if samples are being used for comparative studies (i.e., where the relative photostability of compounds or formulations are being assessed), they should be placed near each other (but not close enough to shade each other, see section “Sample Positioning”) within the cabinet, if possible, although it is generally acceptable to identify a region within the cabinet where the illumination level is *approximately* equal (for example, $\geq 80\%$ of the highest reading) and use this for all samples.

Sample Positioning

It is impossible to deal with every eventuality here but the key factors to consider are as follows:

- Sample depth—UV or visible light will only penetrate a few millimeter into a solid; hence, solid samples should be presented as a thin film (< 3 mm) if possible. Where this is not possible it should be appreciated that it is only the exposed surface that can be affected by the

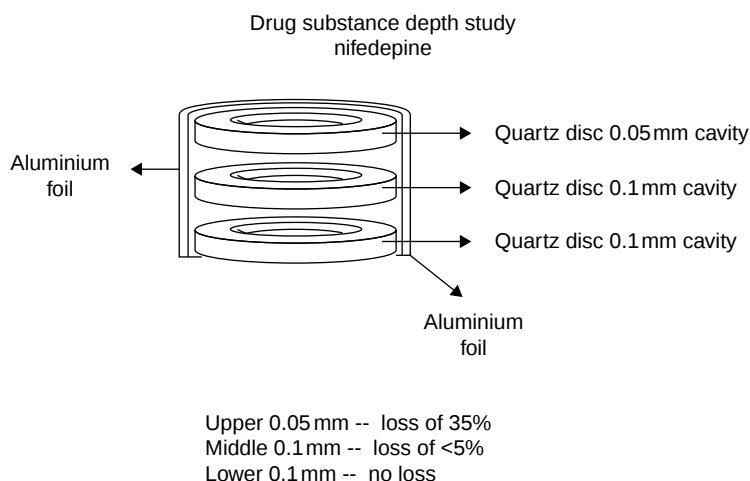


Figure 2 Dependence of nifedepine photodegradation on depth.

light, e.g., the face of a tablet that is pointed toward the light source. Consider this example in Figure 2 (14), which shows the reduction of the extent of photodegradation (35% to no loss) as one moves deeper into the sample (14):

- Maintaining sample position—many cabinets have cooling fans. The air circulation caused by these fans can be strong enough to physically move samples within the cabinet leading to uncontrolled exposure. It is therefore important to fix the samples under test in position in such circumstances. However, the fixing method must not shade the sample in any unintentional way nor have a detrimental chemical effect. Often double-sided adhesive tape is sufficient to secure samples with no adverse effect on the test.
- Sample shading—if samples are placed too close together in the cabinet they can shade each other from full light exposure or (if highly reflective) increase the irradiance received by a sample. One particular danger here is from any foil wrapped control samples, which may add to the general reflectivity within the cabinet. In addition, if the sample is highly colored (or becomes so during the course of the experiment) it can affect the spectral power distribution experienced by the samples around it due to selective reflectance/transmittance of the input light radiation. As a rule of thumb, samples should be placed at a distance apart that is at least twice their height.
- Bulky samples and those of unusual shape—such as infusion bags, tilting tablets, etc.—should be placed to expose the maximum surface area to the light source unless there is a valid reason to do otherwise.

Sample Height

The power from a light source decreases as a square of the distance from it. Thus, if a sample is closer to the light source, it will experience greater irradiance than one further away from it. Within a small sample chamber such differences in power can be significant. Consider Figure 3 (14),

The double volume brings the surface of the sample closer to the light source leading to more degradation in the same unit time due to the increased illumination. As shown by the significant relative loss seen in the horizontal sample, surface area is also important. Obviously, care must be taken when comparing data from experiments conducted using different methodologies. When establishing the test method it is important to consider how the product will be used.

Container

Here it is necessary to consider the deliberate and inadvertent effects of packaging. Let us look at inadvertent effects first.

- It is often necessary to put samples in some sort of holder in order to place them securely in the cabinet. For example, liquid samples will need to be contained for handling reasons while powder ones may need to be contained for logistical and safety reasons. Incorrect choice of container can lead to reduction of incident light power at the sample. To avoid this entirely, samples *could* be presented in quartz cells (which are typically fully transparent >200 nm). However, this is not always practicable and may not be relevant to “real life” conditions. In such cases, thin walled type I (boro-silicate) glass containers are highly transmissive for liquid samples. For solid samples, transparent food wrap film does attenuate light to some extent but is quite reproducible assuming that the same manufacturer of wrap is used. Transmittance spectra of different plastic wraps can be found in a book chapter by Baertschi and Thatcher (15). Even so, before using any particular wrap for the first time, it is wise to run a transmission spectrum so that any effects on sample exposure can be understood.
- Containers with caps can shade samples—consider the following represented in Figure 4 (16). The stability of the compound is apparently inverse to the incident light based on the power map presented in Figure 1 above. For comparison, the light power passing through the capped vials is plotted in Figure 5.

From Figure 5, it becomes clear that the caps shadow the container content far more in the center of the cabinet where the light source is close to perpendicular to the cap compared with nearer the cabinet walls where reflected light can enter the vials.

Now let's turn to deliberate packaging.

- Packaging will hold solid or highly viscous sample(s) in a particular orientation. Since it is the container contents next to the wall that receive the highest light exposure (cf. Fig. 4), it is these that are of most interest. It is therefore necessary to isolate them for testing. One example is shown here (Fig. 6) for tablets in a bottle, where the proportion of the samples near the container wall have been maximized (14).

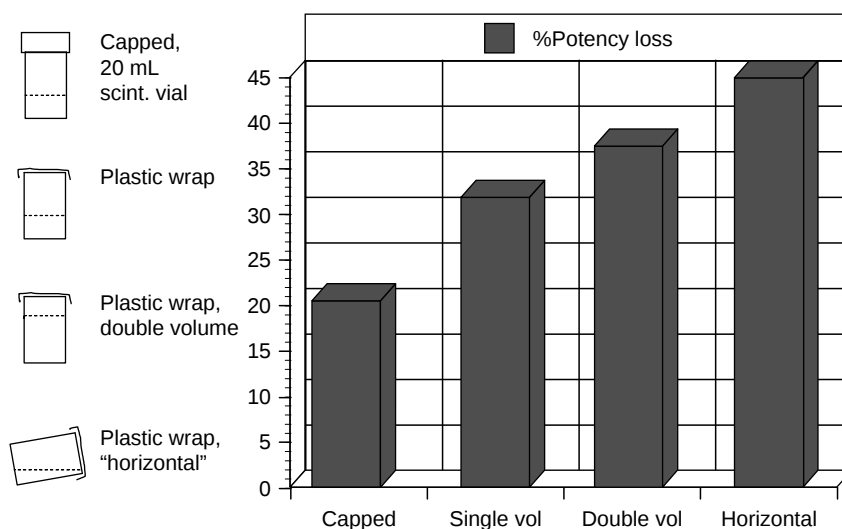


Figure 3 Potency loss due to sample volume.

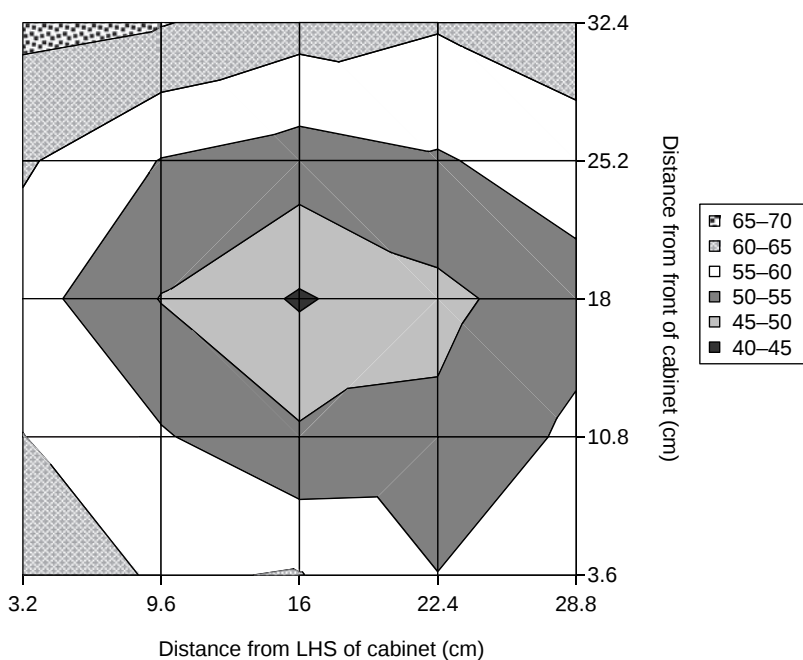


Figure 4 Percentage potency loss of compound Y in capped vials at different positions in an Option 1 light cabinet.

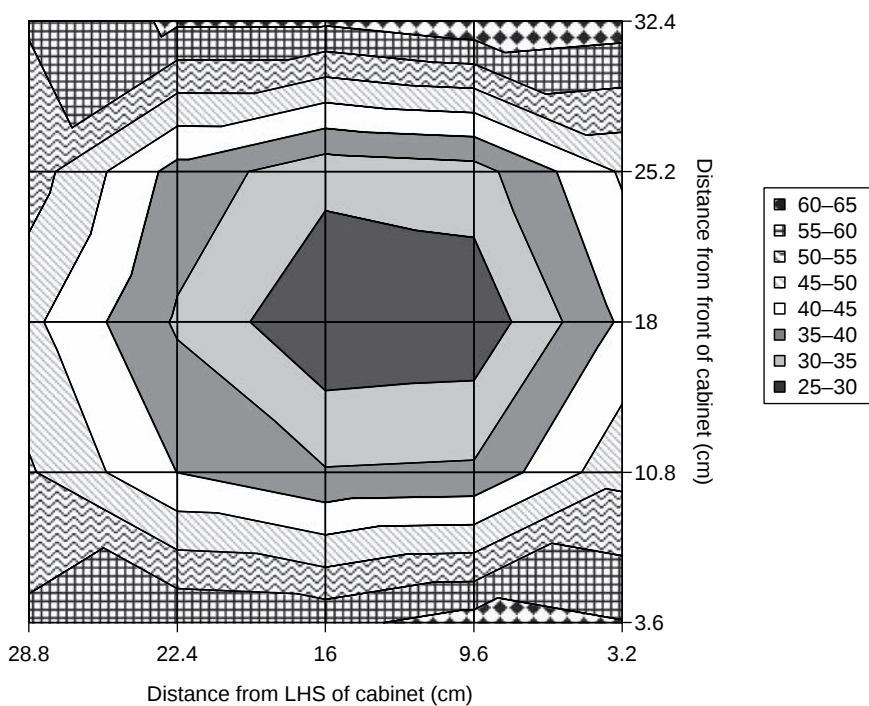


Figure 5 Percentage of maximum available light transmitted through a 14 mL clear glass screw capped vial (16).

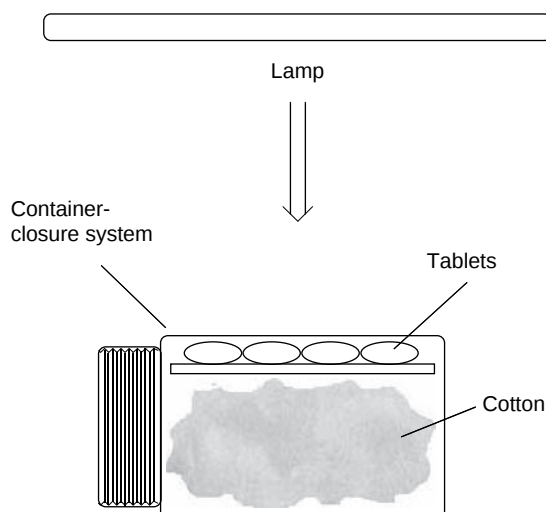


Figure 6 Consideration of effect of packaging on light exposure.

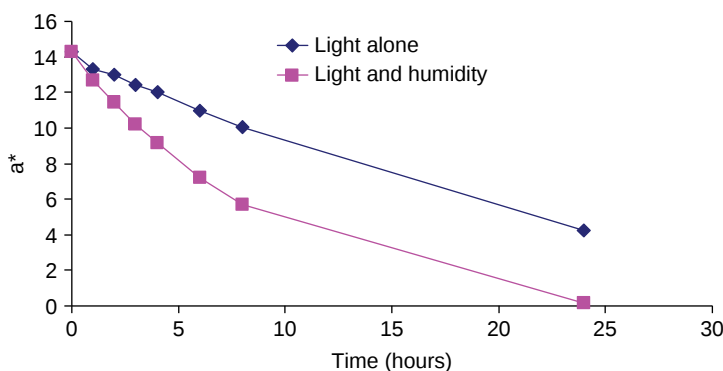


Figure 7 Carmine film coated tablets—light exposed with and without moisture (D. Clapham, unpublished data).

- Packaging also creates a particular microenvironment—for example, a particular relative humidity and heat profile. In particular, covering a sample with an impermeable, light transmissive material may lead to a high local humidity (and possibly temperature) which may “melt” or otherwise malform the capsules physically or chemically. This would not be expected to occur in any realistic exposure environment and can be ameliorated by providing small holes in the cover to allow gaseous exchange.

Effect of Humidity

Many photoreactions are influenced by the presence of moisture and hence by the relative humidity of the environment; for example, this color change (Fig. 7) where a^* is a measure of the redness of the coat.

Kakinoki et al. (30) have shown that the discoloration of famotidine in the solid state in the presence of titanium dioxide, which is present in many film coats, is strongly dependent on relative humidity. In this study, the discoloration rate constant was linearly related to humidity.

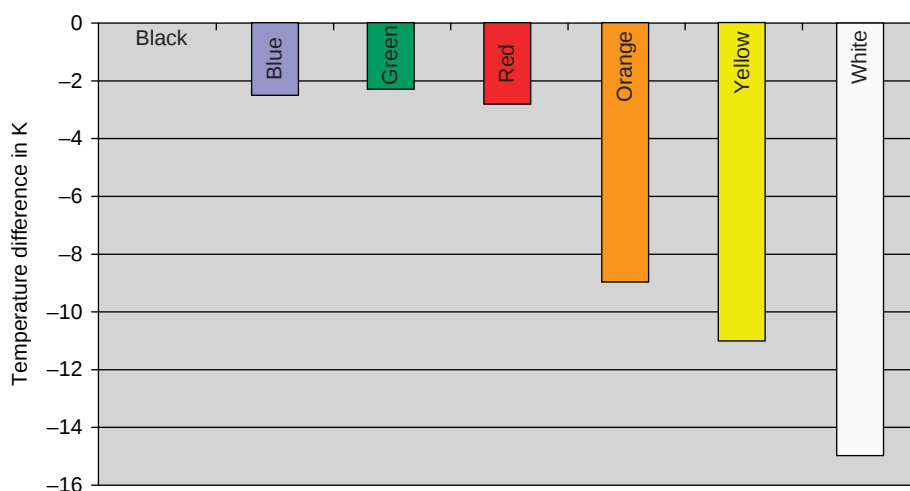


Figure 8 Typical temperature difference between black platinum thermometer and colored samples (20).

Other authors have shown photophysical effects of light exposure. For example, Singh et al. demonstrated that exposure to UV and visible light accelerated the cross-linking of gelatin in formulations leading to a slowing of the dissolution rate of the API from the formulation (17). Singh and Bhutani et al. have also demonstrated that exposure to light can accelerate the rate of moisture pick up of the hygroscopic antitubercular drug ethambutol (18,19).

The microenvironment within a cabinet can be more or less humid than would be the case in normal storage due to the heating effects caused by the light or the method of cooling employed. Thus, it is necessary to know (and where possible control) the humidity in the chamber. When there is no direct humidity control provided by the cabinet itself, an alternative is to include a vial containing a saturated salt solution of the appropriate humidity with the samples or a tightly capped vial that would help to evaluate the combined effect of temperature and humidity.

Effect of Heating

Light is energy and thus when light is absorbed, some of it will dissipate as heat. The extent of heating will depend on the color of the sample. This color may be deliberate (e.g., due to the presence of pigments in a film coat) or inadvertent (e.g., due to color change as a result of degradation). In the above figure (Figure 8), the surface temperature of samples of different color is shown compared to that of the “black panel” thermometer that is often used to monitor the temperature within a sunlight simulation cabinet. The temperature difference is given in degrees absolute (K). Thus, a white sample (as many pharmaceutical tablets are initially) may be up to 15°C cooler than indicated by the chamber temperature display; however, once it begins to yellow the temperature will increase, potentially accelerating the degradation.

Heating needs to be considered when deconvoluting the thermal and photo-induced degradation effects within a test. It should also be remembered that heating can have pronounced physical effects. For example, these may include, changing the viscosity of a semi-solid sample, rendering API amorphous from an initial crystalline state (or vice versa) or softening capsule shells. When interpreting photostress test results, one needs to consider whether these effects may be an artifact of the test conditions.

INTERPRETATION OF STRESS TEST RESULTS

When interpreting stress testing results, one must consider both analytical testing and the implications for product development.

Analytical Testing

Although purely chemical assays involving, e.g., chromatography are clearly valuable tools in the context of photostability, it is important to avoid limiting the evaluation to only these assessments. While chemical degradation is the most likely outcome of API or drug product exposed to light, other physical changes in the solid state may be possible as well (7,10,11,21–27). Thus, a full spectrum of potential chemical and physical changes to the API and formulated product should be considered to allow for a complete understanding of the relative contributions of various photo-driven changes so that they can be managed effectively.

Table 2 highlights several potential chemical and physical changes that can result from photosensitivity that could be considered in a photostress test. It is not intended as an exhaustive list.

The question may arise “How much change in these various aspects is permissible?” The Q1B guidance makes the open-ended statement that, “Acceptable change is change within limits justified by the applicant.” (9) Thus, it is important to view the results of any stress test from the perspective of how the drug substance or drug product will be used and employ the results to develop realistic specifications. Any change that adversely affects quality, safety, and/or efficacy is unacceptable. Some obvious unacceptable changes (or at least changes which will require further investigation) include

1. loss of active with concomitant loss in efficacy;
2. changes which can affect bioavailability (e.g., change of form and isomer);
3. formation of toxic photodegradants;
4. formation of stable products with different pharmacological activity than the parent drug;
5. physical changes which lead to a patient perceptible adverse change (7,25,28) and
6. changes which reduce the ability to reproducibly manufacture the API or DP.

Table 2 General Tests to Consider when Evaluating Photosensitivity

Test	Drug Substance	Drug Products	
		Sterile/Liquids	Oral Solid
Appearance	✓	✓	✓
Impurity/potency assay	✓	✓	✓
Chirality	✓	✓	✓
Color	✓	✓	✓
Particulates (Nephelos/HIAC)	✓	✓	
Preservatives		✓	✓
Flavorings/functional excipients		✓	✓
pH		✓	
Osmolality		✓	
Packaging changes		✓	✓
Resuspendability		✓	
Reconstitution time		✓	
Viscosity		✓	
Hardness			✓
Friability/disintegration			✓
Dissolution			✓

A very conservative position to take with regard to justifying chemical changes would be to follow other ICH guidance covering the content and qualification of impurities (Q3A and Q3B for drug substances and drug products, respectively) at all phases of drug development. However, justification of larger changes is possible and even potentially advantageous, particularly at the earliest stages of development. For example, the existence of photodegradants in formulations used in safety studies (and consequently dosed in relatively large quantities) provides a basis for justifying and qualifying the appearance of these compounds in later clinical materials, assuming of course that the studies demonstrate that they are not particularly toxic.

Implications for Product Development

Although the focus of this chapter is photostress testing, it is important to be able to relate results found in stress tests to the in-use environment in order to understand the possible implications for the product and the patient. To complete the picture, it is important to remember a few other issues in relation to photostability testing.

Generally, the photoreactivity of drug substances or drug products follows the order: solutions > suspensions/dispersions > solids. This is typically attributed to the increased mobility or diffusion of an API in the excited state or to photochemistry requiring solvent participation. Therefore, the effect of any sample manipulation used to facilitate the stressing of the sample needs to be considered. Further, some reaction mechanisms are different in the solid state than in solution. As a general rule, reactions in solution simply occur faster than those in the solid state due to the ease of molecular mobility in solution. However, it is also possible that different reaction mechanisms may operate as well. For example, menadi-one undergoes a [2+2] dimerization in the solid state, but in ethanol solution yields an epoxide as the major product (29). Therefore, the assessment of photostability should take into account the route of manufacture and the likely in-use handling. This may require different photostress testing approaches, such as exposure to lamps with different spectral features, or even exposure as a solution, suspension, or dry powder (for drug substance). The concentration of the drug in a suspension/solution must be carefully considered because as the concentration increases in solution, a smaller percent loss may be observed due to the amount of light to which the total sample is exposed (very little light gets transmitted through the center to the back of the sample). Further, the effect of any organic solvent used to aid the solubilization of the molecule must be considered so that one is not misled by nonrepresentative or nonrealistic photoinitiated reactions. To reduce this likelihood, it is sometimes advisable to compare results from a number of different co-solvent systems (where cosolvents are needed).

When assessing a drug product, both the API and the excipients can contribute to the overall reaction. In order to differentiate changes unique to excipients versus active ingredient, it is recommended to expose a placebo alongside the active formulation. While most excipients are not chromophoric, it is possible to have a photoreaction involving excipients since excipients do contain functional groups that can react with API excited states. However, it is uncommon that a formulation excipient can photoreact and lead to degradation of an otherwise stable API. It is therefore vital to test *both* the API *and* the DP rather than extrapolating from results for the API alone. Another point to consider is that by using a higher power of light to shorten the irradiation (exposure) time, it is possible that different levels of degradation are obtained. This is demonstrated in Figure 9 (20). The reasons for such behavior can be complex.

Analogous to this situation is one where the same apparent total power from two different light sources produces a different level of degradation as shown in Figure 10 (21).

In this case, the fluorescent lamp was particularly rich in a causative wavelength, which resulted in the bleaching of the pigment.

Photo-induced degradation and that from other sources may be cumulative, particularly if the degradation products are the same. As shown in Figure 11 (10), as the amount of

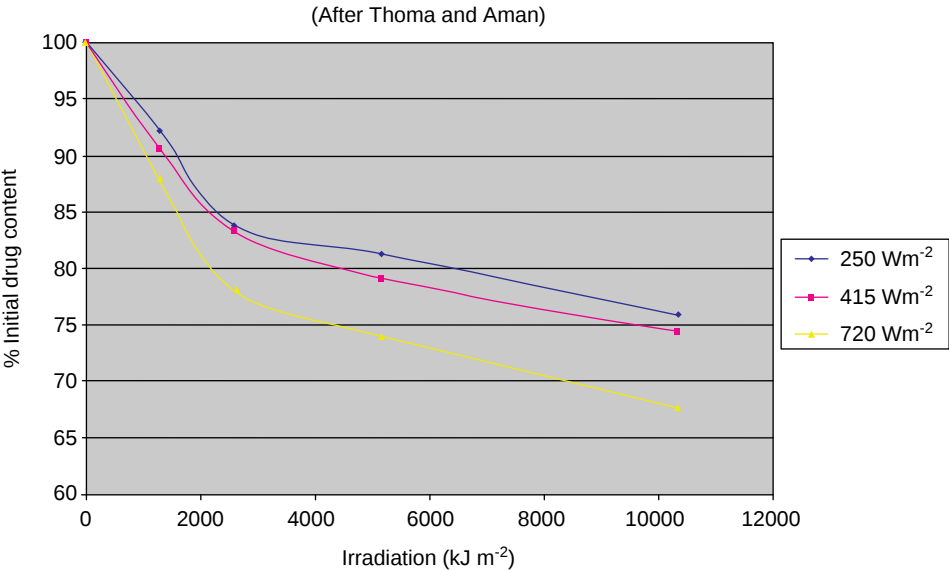


Figure 9 Influence of irradiance on the photostability of Molsidamine 4 mg tablets.

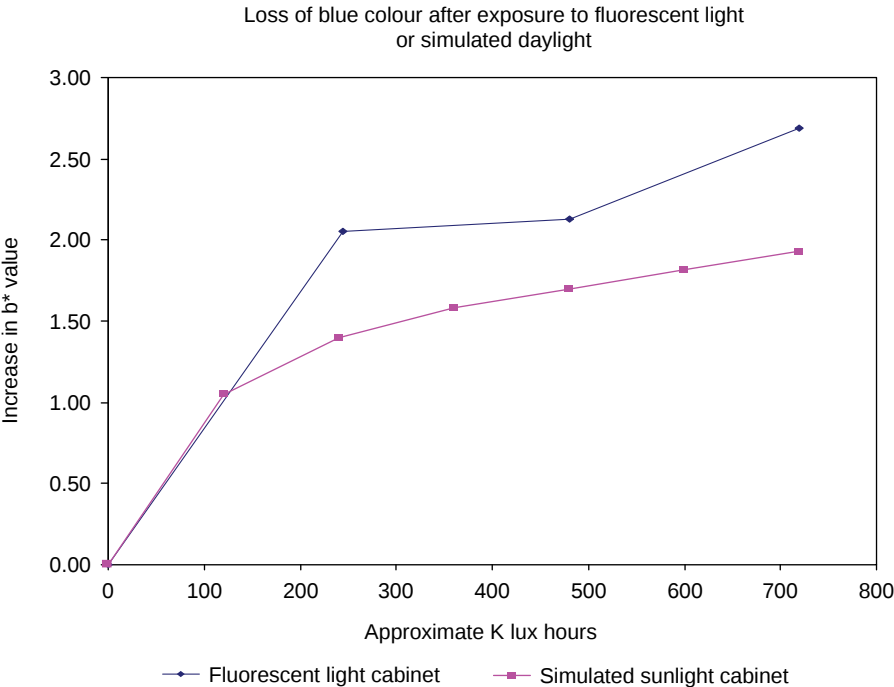


Figure 10 Influence of two different light sources on the loss of blue color 'b' from a film coat.

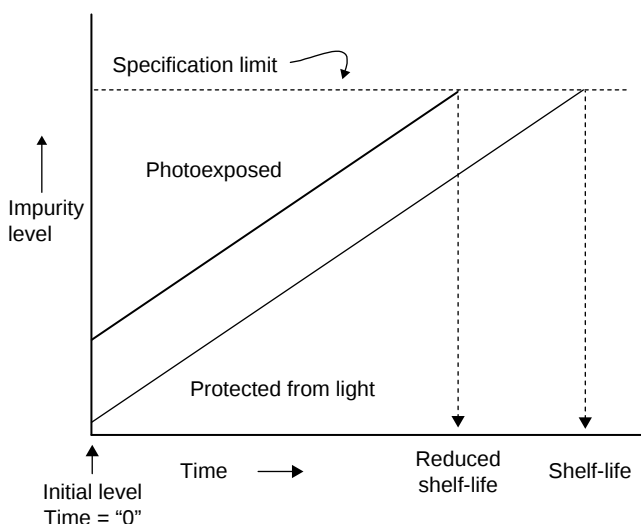


Figure 11 Cumulative effect of photoinstability on shelf life.

photodegradant increases, the shelf life of the product must be reduced in the absence of light protection, (10) because the product will no longer meet the specification.

CONCLUSION

This chapter has covered practical issues to be considered when undertaking photostability stress testing of API and drug products. A well conducted test will allow the pharmaceutical scientist to understand how the molecule and its products respond to irradiation with the different sorts of light source that they will meet during product development and use. This goes well beyond a simplistic compliance with the ICH Q1B guidance. Gaining this understanding is important to the safe and effective use of the active ingredient and its formulations with benefits for both the pharmaceutical company and the patient.

REFERENCES

1. Piechocki JT, Thoma K, eds. *Pharmaceutical Photostability and Stabilization Technology*. Informa Healthcare, 2007.
2. Tonnesen HH, Ed. *Photostability of Drugs and Drug Formulations*. 2nd ed. CRC Press, 2004.
3. Albini A, Fasani E. *Drugs: Photochemistry and Photostability*. Spec. Publ., -R. Soc. Chem., 1998; 225: 326.
4. Tonnesen HH, ed. *The Photostability of Drugs and Drug Formulations*. 1st International Meeting on Photostability of Drugs. Oslo: Norway, 1995,1996.
5. Kleinman MH, Smith MD, Kurali E, et al. An evaluation of chemical photoreactivity and the relationship to phototoxicity. *Reg Tox Pharmacol* 2010; 58(2): 224–32.
6. Onoue S, Tsuda Y. Analytical studies on the prediction of photosensitive/phototoxic potential of pharmaceutical substances. *Pharm Res* 2006; 23(1): 156–64.
7. Templeton AC, Xu H, Placek J, Reed RA. Implications of photostability on the manufacturing, packaging, storage, and testing of formulated pharmaceutical products. *Pharm Technol* 2005; 29(3): 68–86.
8. Lynch AM, Smith MD, Lane AS, et al. An evaluation of chemical photoreactivity and the relationship to photogenotoxicity. *Reg Tox Pharmacol* 2010; 58(2): 219–23.
9. ICH. *Stability Testing: Photostability Testing Of New Drug Substances And Products (Q1B)*. ICH Harmonized Tripartite Guideline, 1996.

10. Thatcher SR, Mansfield RK, Miller RB, Davis CW, Baertschi SW. Pharmaceutical Photostability: A technical guide and practical interpretation of the ICH guideline and its application to pharmaceutical stability – part I. *Pharm Technol North Am* 2001; 25(3): 98–110.
11. Drew HD. Photostability of drug substances and drug products: a validated reference method for implementing the ich photostability study guidelines. *Spec Publ – R Soc Chem* 1998; 225(Drugs: Photochemistry and Photostability): 227–42.
12. Tashtoush BM, Jacobson EL, Jacobson MK. UVA is the major contributor to the photodegradation of tretinoin and isotretinoin: implications for development of improved pharmaceutical formulations. *Int J Pharm* 2008; 352(1–2): 123–8.
13. Tonnesen HH, Baertschi SW. The questions most frequently asked. In: *Photostability of Drugs and Drug Formulations*. 2nd ed. Boca Raton: CRC Press, 2004, p. 161–72.
14. Baertschi SW. Pharmaceutical Photostability Testing: Sample Presentation. *Pharmaceutical Photostability*. PPS 04, London: UK, 2004.
15. Baertschi SW, Thatcher SR. Sample presentation for photostability studies: problems and solutions. In: Piechocki JT, Thoma K, eds. *Pharmaceutical Photostability and Stabilization Technology*. New York: Informa Healthcare USA Inc., 2007, pp. 177–201.
16. Clapham D, et al. The Importance of Sample Positioning and Sample Presentation within a Light Cabinet. PPS 01. NC: Research Triangle Park, 2001.
17. Singh S, Manikandan R, Singh S. Stability testing for gelatin-based formulations: rapidly evaluating the possibility of a reduction in dissolution rates. *Pharm Technol* 2000; 24(5): 58, 60, 62, 64, 66, 68, 70, 72.
18. Singh S, Bhutani H, Mariappan TT, Kaur H, Bajaj M, Pakhale S. Behavior of uptake of moisture by drugs and excipients under accelerated conditions of temperature and humidity in the absence and the presence of light: 1. Pure anti-tuberculosis drugs and their combinations. *Int J Pharm* 2002; 245 (1–2): 37–44.
19. Kaur H, Mariappan TT, Singh S. Behavior of uptake of moisture by drugs and excipients under accelerated conditions of temperature and humidity in the absence and the presence of light: Part III. Various drug substances and excipients. *Pharm Technol* 2003; 27(12): 52, 54, 56.
20. Boxhammer J, Schoenlein A. Technical requirements and equipment for photostability testing. In: Toennesen HH, ed. *Photostability of Drugs and Drug Formulations*. 2nd ed. Boca Raton: CRC Press; 2004: 111–36.
21. Aman W, Thoma K. ICH guideline for photostability testing: aspects and directions for use. *Pharmazie* 2003; 58(12): 877–80.
22. Anderson NH, Byard SJ. Photostability testing: design and interpretation of tests on new drug substances and dosage forms. In: *Photostability of Drugs and Drug Formulations*, 2nd edn. CRC Press; 2004: 137–59.
23. Helboe P. The elaboration and application of the ICH guideline on photostability: a European view. Special Publication—Royal Society of Chemistry 1998; 225(Drugs: Photochemistry and Photostability): 243–6.
24. Sequeira F, Vozzone C. Photostability studies of drug substances and products. *Pharm Technol* 2000; 24(8): 30, 32, 34–5.
25. Templeton AC, Bowen WE, Klein LJ, Harmon PA, Lu Y, Reed RA. Unexpected photochemistry in pharmaceutical products: a review on the role of diluents, excipients, and product components in promoting pharmaceutical photochemistry. *Drugs Pharm Sci* 2007; 163: 223–51.
26. Thatcher SR, Mansfield RK, Miller RB, Davis CW, Baertschi SW. Pharmaceutical Photostability. A technical guide and practical interpretation of the ICH guideline and its application to pharmaceutical stability: part II. *Pharm Technol North Am* 2001; 25(4): 50–62.
27. Toennesen HH. The international conference on harmonization photostability guideline: a discussion of experimental conditions. *Drugs Pharm Sci* 2007; 163(Pharmaceutical Photostability and Stabilization Technology): 47–60.
28. Reed RA, Harmon P, Manas D et al. The role of excipients and package components in the photostability of liquid formulations. *PDA J Pharm Sci Technol* 2003; 57(5): 351–68.
29. Albini A, Fasani E. Rationalizing the photochemistry of drugs. In: Toennesen HH, ed. *Photostability of Drugs and Drug Formulations*. 2nd ed. Boca Raton, FL: CRC Press; 2004; 67–110.
30. Kabinoki K, Yamane K, Teraoka R, Otsuka M, Matsuda Y. Effect of relative humidity on the photocatalytic activity of titanium dioxide and photostability of famotidine. *J Pharm Sci* 2004; 93(3): 582–9.

9 | Role of “mass balance” in pharmaceutical stress testing

Mark A. Nussbaum, Andreas Kaerner, Patrick J. Jansen, and Steven W. Baertschi

INTRODUCTION

The assessment of degradation in pharmaceutical products involves two aspects of analytical measurement. First, a selective analytical method must be available for accurate assay of the parent drug compound, in order to correctly measure any loss. Second, methodology should be in place for quantification of the degradation products formed. Ideally, when degradation occurs, the measured amount of parent drug lost should correlate well with the measured increase in degradation products. This correlation is referred to as “mass balance” (1). More recently, the International Conference on Harmonization (ICH) has provided a definition of “mass balance” as follows:

The process of adding together the assay value and levels of degradation products to see how closely these add up to 100% of the initial value, with due consideration of the margin of analytical error (2).

Clearly, from the law of conservation of matter, any true decrease in the mass of parent compound (and other reactants) is necessarily equivalent to the total mass of all degradation products formed. In a closed system, mass balance would thus be assured if one could accurately quantify all species present in the original and degraded material. However, such is almost never the case. The requirement for a “closed system” is rarely met. For example, degradation may produce volatile substances that escape from the sample matrix. Adsorption or other physical losses may also result in inaccurate assessment of the amount of material degraded or produced. In other words, it is not typically practical to analyze a sample’s entire environment (e.g., container, atmosphere, etc.). Finally, one may deliberately choose not to quantify certain degradation products if the given degradation pathway can be monitored by assessing a limited number of key substances. As always, the analyst must balance time and resource demands to provide the information necessary to understand degradation without going to extreme measures to quantify components of little interest (3).

WHY IS MASS BALANCE IMPORTANT?

Mass balance in pharmaceutical analysis is important for several reasons. By demonstrating that degradative losses of parent drug correlate well with the measured increase in degradation products, mass balance provides evidence that there are no significant degradation pathways unaccounted for. It should be noted that this evidence need not encompass every degradation fragment; it is possible for a portion of a parent drug (e.g., a nonchromophoric moiety) to be lost during degradation without being detected at all. In other words, a particular degradation product may be equally invisible to both the parent drug assay and the degradation product method. Fortunately, such cases are likely to cause a change in chromatographic retention of the degraded parent drug, since its structure will have changed. Thus, while one degradation product may go undetected, its presence can be inferred by the detection of the “other piece” of the parent drug that still contains the detectable moiety. Thus, the particular degradation pathway may still be tracked and molar mass balance still achieved. Conversely, if one observes, for example, a 20% loss of parent drug but only measures a 5% increase in degradation products, it is likely that degradation products are formed that are not accurately determined by the given method(s). Because degradation products could potentially be toxic or otherwise compromise the safety of the drug, it is important to have methods that track all major degradation products and/or pathways. Thus, safety is the primary reason for evaluating mass balance (2–4).

Mass balance is also important in understanding alternative degradation pathways (3–5). For example, consider a situation where both acid-catalyzed degradation and oxidative degradation produce substantial loss of parent compound in stress-testing studies. If good mass balance is achieved for the acid-catalyzed degradation, but not for the oxidative degradation, further work to better understand the oxidative degradation pathway(s) is warranted. It may be that the poor mass balance in the latter case results from important oxidative degradation products that are unaccounted for or from structures which need to be more fully elucidated to understand response factor differences.

Furthermore, mass balance is useful in method validation (1,3,6–9). In order to demonstrate that analytical methods are stability indicating, unstressed and stressed materials are often compared. An increase in degradation products that correlates well with loss of parent drug helps to demonstrate that the methods can be used to accurately assess degradation. Such correlation then translates into an enhanced ability to predict long-term stability profiles to aid in shelf life determinations.

A caution is in order here. Mass balance is a useful tool, but, of itself, is not a substitute for understanding degradation mechanisms. In cases where only a small portion of the parent compound is detectable (e.g., a single chromophoric moiety in a macromolecule), good mass balance could be quite simply achieved as the parent degrades and the single detectable degradation product increases correspondingly. However, numerous other fragments and rearrangements could go undetected. Alternatively, detection response factors may differ significantly between parent compound and degradation products, causing a false assessment of mass balance. It is therefore important to know the structure of what is being detected in order to gain insight into degradation mechanisms and likely structures of any products that are not being detected. In some cases, it may be necessary to use an alternative detector to further clarify what products are being formed and to what extent. Such information is expected to be understood by phase III of clinical development (3).

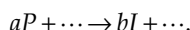
HOW IS MASS BALANCE MEASURED AND EXPRESSED?

Mass balance can be calculated and expressed in a variety of ways. The amount of parent compound lost and degradation products formed can be expressed in terms proportional either to weight or to number of moles. The term “mass balance” is suggestive of a straightforward correlation of mass or weight lost and gained. If all starting materials and degradation products are accounted for, then a correlation in terms of weight is appropriate. However, if degradation involves the formation of products of substantially different molecular weight, or which are not all readily measured, it may be more appropriate to consider molar mass balance.

For the purposes of this discussion, then, the following definitions will be used (examples are given below the set of definitions). Let P be the parent drug and I be the impurity or degradation product. Assume that $M_{p,0}$ and $M_{p,x}$ are the mass of parent compound (and other starting reactants) initially and at time x , respectively; $M_{i,0}$ and $M_{i,x}$ are the total mass of impurities initially and at time x , respectively. Similarly, $N_{p,0}$, $N_{p,x}$, $N_{i,0}$, and $N_{i,x}$ are the analogous number of moles of each.

Mass balance: The situation in which the measured mass of parent and other reactant(s) consumed is equivalent to the measured increase in mass of degradation product(s), that is, $M_{p,0} - M_{p,x} = M_{i,x} - M_{i,0}$.

Molar mass balance: The situation in which the measured increase in moles of degradation product(s) is equivalent to that predicted via a balanced chemical reaction from the number of moles of parent consumed. For a degradation reaction,



molar mass balance at time x can be expressed as

$$(N_{p,0} - N_{p,x})/a = (N_{i,x} - N_{i,0})/b$$

Furthermore, an observed deviation from mass balance can be expressed in either absolute or relative terms, as described below.

Absolute mass balance deficit (AMBD): The difference between the measured amount of parent compound consumed and of degradation product(s) formed, that is, $AMBD = (M_{p0} - M_{px}) - (M_{lx} - M_{l0})$. While AMBD can be expressed in units of mass, it is commonly expressed in percent (see example below).

Relative mass balance deficit (RMBD): The absolute mass balance deficit expressed as a percentage of the total amount of parent consumed, that is, $RMBD = 100\% \times [(M_{p0} - M_{px}) - (M_{lx} - M_{l0})] / (M_{p0} - M_{px})$.

The absolute and relative molar mass balance deficit can be analogously calculated using the number of moles—in place of mass—of parent and degradation product(s).

Example 1: Stress testing studies indicate a loss of parent compound from 100 to 86 µg/mL (a loss of 14.0%), and an increase in total related substances from 2.0% to 11.5% (relative peak area). The AMBD is thus $14.0 - (11.5 - 2.0) = 4.5\%$ (or 4.5 µg/mL). The corresponding RMBD is $4.5/14.0 = 32.1\%$.

Example 2: Stress testing studies indicate a loss of parent compound from 100 to 86 µg/mL (a loss of 14.0%), but no corresponding increase in related substances. Now $AMBD = 14 - 0 = 14.0\%$, and the $RMBD = 14.0/14.0 = 100\%$.

Example 3: Stress testing studies indicate a loss of parent compound from 200 to 180 µmoles/mL (a loss of 10.0%), and an increase in one key degradation product from 2.1 to 15.5 µmoles/mL. The degradation product (*I*) is believed to be formed from a dimerization reaction of the type



Thus, the loss of 20 µmoles/mL of *P* would be expected to produce 10 µmoles/mL of *I*. In this case, the measured increase of *I* exceeds what would be expected from molar mass balance. The AMBD in this case is $[(200 - 180) / 2] - (15.5 - 2.1) = 10 - 13.4 = -3.4 \mu\text{mol/mL}$. The RMBD is therefore $100\% \times (-3.4/10) = -34\%$.

AMBD and RMBD are both zero in the case of perfect mass balance, positive when the measured increase in degradation products is less than the loss of parent, and negative when the measured increase in degradation products exceeds the loss of parent. The RMBD is particularly useful in assessing how significant a mass balance issue is, since it is independent of the extent of degradation (in contrast to AMBD). The RMBD, in other words, expresses the relative inaccuracy of the measured increase in degradation products.

Often, stress-testing results from HPLC assays are reported in terms of percentages lost or gained upon degradation, based on peak areas. Consideration should be given to exactly what the reported percentages mean, in order to understand mass balance. Generally, for stress testing, any counter-ion or other inorganic impurity present is ignored, and percentages are with respect to the total sum of parent compound and related impurities. Also, for solid-state samples, changes in water or other volatile components should be accounted for. One way to address this is to analyze samples using thermogravimetric analysis (TGA) or Karl-Fisher (for water content) to account for loss or gain of volatiles. Another approach is to perform the analyses such that volatiles lost or gained are compensated for in the procedure. This can be accomplished using a method that involves individually weighing samples (from a lot with a known volatiles content) for each time point to be analyzed and placing in individual containers to be placed under the stress condition. At the prescribed time point, the individual container (with a known pre-weighed amount of sample) is completely dissolved and diluted to a specified volume. Thus, any change in volatiles content is irrelevant to the assay result. Thus, when reporting changes in parent content after stress testing, it is important to clearly state what a percentage change refers to, and whether changes in overall mass (e.g., due to water loss or gain) are normalized.

Typically, organic degradation products ("related substances") are determined by HPLC. It is important to know whether degradation products are quantified against an external

standard, or—more commonly—by relative peak area. HPLC peak areas are integrated and the results are, in the simplest case, reported as a percentage of the total of all integrated peaks. Of course, this assumes uniform response factors (e.g., UV absorptivity) for degradation products and parent, and such an assumption may not be valid (10–14). Even when valid, or when response factor corrections are used, it is important to remember what the total of the HPLC peak areas excludes. Clearly, any substances which do not respond to the given HPLC detector are not included in the total. Also, the assumption is made (and presumably tested) that all degradation products are eluted from the column.

Finally, if multiple analytical methods are required in order to quantify all the relevant degradation products, then the meaning of relative peak areas becomes more complex. For example, consider a compound which undergoes racemization in addition to achiral degradation. Suppose compound Y, initially 95% parent and 5% related substances, is also initially purely R-isomer. After stress testing, an achiral assay yields the result of 80% parent, and a chiral assay shows that the parent is now 2:1 R:S. Thus, if we had started with 100 mg of sample, we should now have 20 mg of related substances, and of the remaining 80 mg parent, 53 mg is R- and 27 mg is S-isomer. Overall, then, there has been a 44% loss (by weight) of the R-form of compound Y (95–53 mg). Given mass balance, the related substance assay should show an increase from 5% to 20% relative peak area, and the chiral assay gives an increase from 0% to 33% S-isomer (relative to the total of R- and S-isomers). Hence, it is important that results be clearly stated. For example, in this case, one could report a 20% loss in compound Y (total enantiomers), or a 44% loss of the R-enantiomer of Y after stress testing.

STRESS TESTING AND MASS BALANCE

The relation of mass balance to stress testing is intrinsic to the ICH guidelines. As described by ICH, stress testing “can help identify the likely degradation products, which can in turn help establish the degradation pathways and the intrinsic stability of the molecule and validate the stability indicating power of the analytical procedures used” (2). Thus, an assessment of mass balance is an important part of achieving the goals of understanding degradation pathways and evaluating the capability of the analytical procedures to detect all the relevant degradation products.

When carrying out stress-testing studies during development of analytical methods for a particular drug, there are practical problems to consider. If degradation is observed under some stress condition (as inferred by a loss of parent in the analytical assay), how does one determine whether all of the degradation products are being detected when most, if not all, are unknown? It is the intent of this chapter to provide a practical guide for making this assessment.

It is important to remember that the goal of stress testing is not primarily to achieve mass balance in the analytical results, but rather to achieve a full understanding of the degradation chemistry. That is, if the degradation pathways are fully understood, then it is relatively straightforward to determine whether all relevant degradation products are being accurately determined. Typically, this kind of understanding cannot be achieved unless the structures of the main degradation products are known. Correlation of the degradation product structures with scientifically reasonable pathways then enables one to assess whether or not any major products are unaccounted for. This mechanistically driven approach [also referred to as a chemistry-guided approach (3,15); see chap. 2] can provide an assessment of “the completeness of the investigation of the routes of degradation and the use, if necessary, of identified degradants as indicators of the extent of degradation via particular mechanisms” (16). A practical example of utility of this scientifically guided approach is given below, in the case of compound LY297802 (see section “Negative Mass Balance Deficit”).

CAUSES OF AND APPROACHES TO SOLVING MASS BALANCE PROBLEMS

There are several potential causes of poor mass balance. The list of considerations below covers the most common difficulties and suggests ways to diagnose and resolve them.

Positive Mass Balance Deficit

In these cases, the increase in mass (or number of moles) of degradation products is less than the corresponding decrease in parent. Potential sources and resolution of the problem are described as follows.

Degradation Product(s) Not Eluted from the HPLC Column

There are a number of practical ways to diagnose this problem: (a) the HPLC method can be modified to elute any additional impurities; (b) samples can be analyzed using UV spectrophotometric analysis; (c) samples can be analyzed using the HPLC system without the column present (flow injection analysis); or (d) an alternate/orthogonal separation can be used. One caution here is that these approaches assume that the drug and all degradation products are fully soluble and are detectable by the HPLC detector (e.g., UV absorbance).

- (a) The HPLC method can be modified to elute any additional impurities.
Reversed-phase methods can be modified to elute retained, nonpolar compounds by increasing the strength of the mobile phase or increasing the analysis time. This can be done using either gradient or isocratic elution.
- (b) Stressed, partially degraded samples can be analyzed against a standard using UV spectrophotometric analysis and the results compared to the results obtained by HPLC analysis of the same samples and standard.

This method is useful for HPLC methods that use UV detection. Because UV spectrophotometric analysis involves no separation, there is no chance that compounds are being missed due to retention on a column. Using this approach, a partially degraded sample is dissolved (in the case of solid samples) or diluted (in the case of solution samples) in the mobile phase solvent. The full UV (and/or VIS) spectrum for the partially degraded sample is obtained and compared to the spectrum of the undegraded sample. (If this is a gradient HPLC method, it is recommended that the samples be dissolved in the approximate mobile phase composition under which the parent elutes.) The ratio of the absorbance of the partially degraded sample to that of the undegraded sample is obtained at the wavelength used in the HPLC method. This absorbance ratio is then compared to the ratio of total peak area obtained by the HPLC method for the partially degraded sample compared to the undegraded sample. If all of the impurities are detected with the HPLC method, then the total HPLC peak area from the partially degraded sample divided by the HPLC peak area from the undegraded sample should be equivalent to the absorbance ratio. If the HPLC method utilizes a photodiode array (PDA) detector, comparisons can be determined at multiple wavelengths, if desired. If the HPLC area ratio is significantly less than the spectrophotometric absorbance ratio, then one or more of the degradation products must not be eluting from the column.

- (c) The sample can be analyzed using the HPLC system without the column present (i.e., flow injection analysis).

In this experiment, the column is removed from the HPLC system and the total peak area compared to the total peak area obtained when using a column. If the total peak area obtained with the column present is significantly less than that when the column is absent, then the HPLC method must be missing some of the total mass. In the absence of the column, all related substances and parent compound co-elute, giving a single unretained peak with an area proportional to the total amount of detectable material. The total area of all peaks should be the same when the column is present, assuming all species are eluted. If the total area is less when the column is present, then it is likely that one or more compounds are not eluted under the given conditions. One potential difficulty with this diagnosis tool is the impact of the sample solvent. If significantly different from the mobile phase, the solvent effects on sample response may make accurate integration difficult in the absence of the column. In addition, it is important that the quantity of sample injected

does not produce peak area(s) that are off-scale or outside the linear range. Also, one must keep in mind that any analyte that adsorbs to injection valves, loops, or internal surfaces of the HPLC system would not be detected. Finally, if a mobile-phase gradient is used when the column is present, the changing solvent composition can affect analyte response and, consequently, the peak area of eluting species. One can determine if the gradient has such an impact by comparing the total areas obtained, without the column, when each extreme of the gradient mobile-phase composition is used isocratically.

- (d) An alternate/orthogonal separation can be used and the results compared to the HPLC results.

The use of reversed-phase thin layer chromatography (RP-TLC) can aid in the investigation by revealing slowly migrating compounds or compounds at the origin. The orthogonal techniques of normal-phase HPLC, normal-phase TLC, and capillary electrophoresis (CE) can also be powerful investigative tools in determining whether the original HPLC method fails to elute all products (1).

Degradation Product(s) Not Detected by the Detector Used

Ultraviolet absorbance is the most common detection technique for HPLC. Although widely applicable, UV detectors do not detect all compounds. Degradation may produce compounds without chromophores, in which case the observed increase in degradation products will be smaller than the loss in parent compound. The diagnosis (as well as the solution) for the problem may be to use a shorter wavelength or an alternative detector (e.g., evaporative light-scattering detection (ELSD), refractive index (RI), conductivity, charged-aerosol detection (CAD), mass spectrometry (MS), or flame ionization detection (FID)). It is important to keep in mind that most such detectors, while broadly applicable, are—like UV—not uniform in their response. For example, compounds with significant vapor pressure give poor response by ELSD and CAD, and MS response varies greatly with ionizability. However, such detectors can be very useful in confirming the presence of degradation products undetected by the original method (14–24).

As an example of this type of mass balance issue, consider drug candidate LY297802 (Fig. 1). Stress testing of aqueous solutions of LY297802 under cool-white fluorescent lighting (approx. 17,000 lux) produced a 5.4% loss after 3 days and a 42% loss after 7 days (overlaid chromatograms, Fig. 2). However, the corresponding increases in degradation products were only 0.4% and 1.3%, respectively, by the gradient HPLC–UV-related substance method, revealing a significant analytical mass balance deficit. Careful inspection of the containers in which the samples were exposed revealed an insoluble hazy film deposited on the surfaces. Collection of this insoluble film and subsequent analysis by probe EI–MS revealed that the film was elemental sulfur (S_8). As the sulfur was likely originating from degradation of the thiadiazole ring, the potential for formation of nonchromophoric products was considered. LC–MS analyses of the solutions were unfruitful, and the potential for formation of volatile products was considered. Hexane extraction (of the aqueous light-degraded solution, basified to give the free base of LY297802) followed by GC–FID revealed two major degradation products in the degraded samples. Analysis using GC–MS (with accurate mass measurements) provided molecular

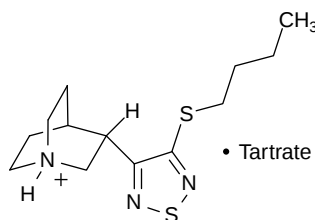


Figure 1 Structure of LY297802.

formula information. As a result, structures were elucidated, providing an understanding of the degradation chemistry and the reason for the lack of detectability with HPLC–UV (i.e., the degradation products were volatile and nonchromophoric) (25).

Degradation Product(s) Lost from the Sample Matrix

In some cases, degradation products are inadvertently excluded from the sample tested because of insolubility, volatility, or adsorption losses. Instances of insolubility are usually the most obvious and straightforward to solve. In such cases, visual observation or turbidity measurements may reveal the problem. Use of a different sample solvent or isolation and specific testing of the insoluble material may then be necessary. For example, in the degradation of compound LY297802 described earlier (25), some insoluble material was observed and determined to be elemental sulfur, providing a valuable clue to the mass balance problem. Of course, insoluble degradation products are less obvious when present in drug products containing insoluble excipients. Isolation and examination of the insoluble material, compared to placebo or undegraded drug product, is appropriate in such cases.

If degradation products are lost because of their volatility, other analytical techniques can be utilized. In such instances, it may be appropriate to extract the sample using solvent–solvent extraction (as in the LY297802 example mentioned earlier) or to degrade material in a manner in which the headspace is captured. Gas chromatography (GC–FID or GC–MS) can then be used to compare the headspace from degraded and undegraded samples. In limited cases, if the degradation is thermally induced and rapid, TGA with associated vapor spectral analysis (e.g., IR and MS) may be a useful approach. Finally, if the degradation can be generated at low temperatures, it may be possible to minimize volatility simply by keeping the sample cold.

Degradation products may also adsorb to the sample container or to insoluble excipients. In the latter case, the diagnosis and resolution of the problem is the same as if the degradation product were insoluble (see above). If adsorption to the container is a potential issue, then the most straightforward approach is to compare results using different container materials (e.g., glass and polypropylene). In some cases, modifying the container surface or changing the sample solvent (e.g., pH and solvent strength) may be necessary to minimize the adsorption. Occasionally, particularly strong solvents may be required to remove the adsorbed material.

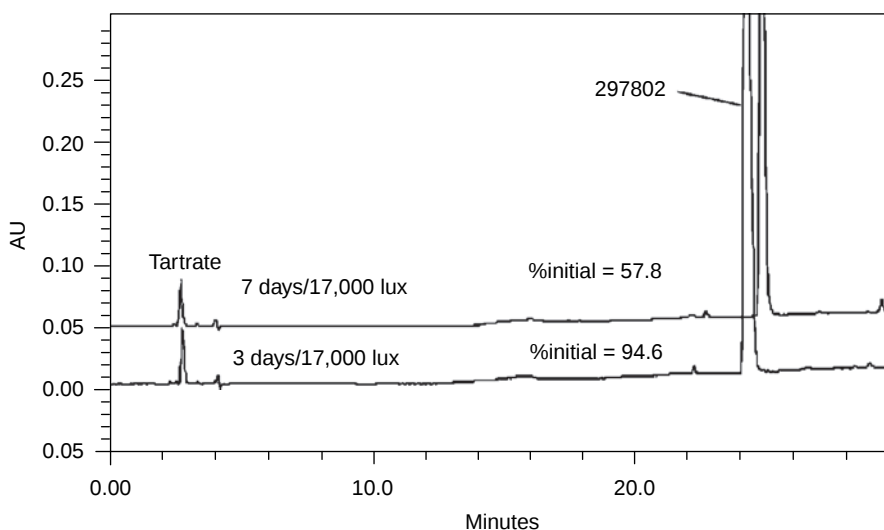


Figure 2 HPLC-related substance chromatograms (UV detection) of 297802 tartrate aqueous solution exposed to cool-white fluorescent light (17,000lux) for 3 and 7 days.

Parent Compound Lost from the Sample Matrix

In rare cases, the parent compound may itself be lost from the sample matrix due to volatility or adsorption. Often, even if such losses occur, they will be insignificant in proportion to the quantity of parent present. However, if significant, the decrease in assay results would not be due to degradation and so would not correspond to any increases in degradation products. Generally, information obtained prior to stress testing (e.g., melting and/or boiling point, vapor pressure, and tendency to adsorb to various materials) should provide indications of this potential problem. Diagnosis and resolution are approached as described above.

Degradation Product(s) Co-eluting with the Parent Compound in the Related Substance Method

If a degradation product co-elutes with the parent compound in both assay and related substance methods, then the resulting mass balance depends on the response factor of the impurity relative to that of the parent compound under the given conditions. If the impurity formed has a smaller response factor, then there will be a positive mass balance deficit. If the response factor of the impurity is greater than that of the parent, then there will be a negative mass balance deficit. If the response factors are the same, then no mass balance deficit will result. Examination of the parent compound peak by photodiode array (PDA)–UV detection (and using UV-homogeneity algorithms) or by MS detection may reveal the co-eluting impurity as peak heterogeneity. Of course, PDA detection is effective only for impurities that have distinct UV spectra and that do not perfectly co-elute with the parent. In addition, the sensitivity of PDA to detecting peak heterogeneity is dependent on how different from the parent the spectral properties of the impurity are. For some related substances, PDA detection may be unable to detect impurities at levels below a few percent. LC–MS may provide greater sensitivity to a wider range of potential co-eluting impurities; however, LC–MS is more expensive and more restrictive in the types of mobile phase that can be used. Alternatively, an orthogonal separation technique (e.g., CE) can be used to check for co-eluting impurities. If a co-elution problem is discovered, the related substance method should be modified to separate the co-eluting impurity.

Degradation Products are Not Integrated Due to Poor Chromatography

Some degradation products may not chromatograph well (e.g., due to adverse interactions with trace metal impurities or residual silanols, on-column conversion from one product to another, etc.) and, although they may elute from the HPLC column, the resulting broadened peaks (often severely tailing or fronting) can easily be “missed” and remain unintegrated, especially when the broad peak area is present at low levels. Alternatively, if the parent drug degrades to a large number of products that are poorly resolved, the chromatogram observed upon analysis may not reveal discrete peaks but rather an elevated baseline. At low levels, such an elevated baseline can easily escape detection. Situations such as this are not uncommon, especially when isocratic HPLC methods are used. Running a blank (and overlaying the chromatogram) can be very helpful in determining if a baseline elevation is from the sample or simply an artifact of the chromatography. Experiments to determine if low mass balance results are from poor chromatography are the same as those that would be performed to determine if degradation products are not eluting from the column [see section “Degradation Product(s) Not Eluted from the HPLC Column”].

An example of a drug that degrades to numerous poorly resolved species is shown in Figure 3. In this example, a partially degraded sample was analyzed by RP-HPLC (with a relatively “shallow” gradient of 5–70% acetonitrile over 25 min), and a number of known degradation products, with known response factors, were detected, but the RMBD was 69.1% (83.5% assay and 5.1% total impurities). The possibility of degradation products not eluting from the column was considered, and an experiment was designed to test this hypothesis. In this experiment, the stressed material was assayed without a column in place (flow injection). If all of the degradation products were detected with the RP-HPLC method, then the sum of the HPLC assay and related substance results (i.e., $83.5 + 5.1 = 88.6\%$) should’ve been equivalent

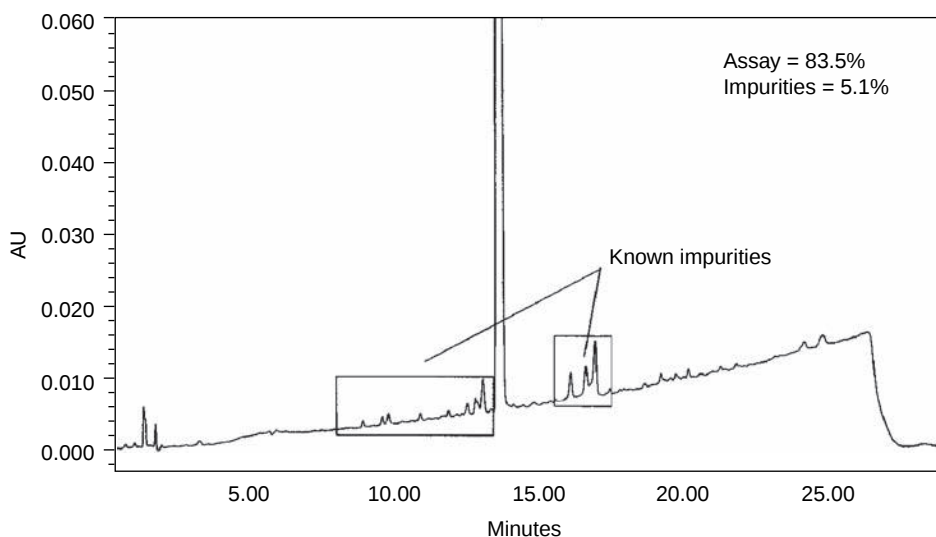


Figure 3 HPLC chromatogram obtained on a thermally stressed drug product using gradient HPLC (“shallow” gradient method).

to the flow injection result. The flow injection result was 97.3% indicating that a significant amount of the total mass (degradation) was not being detected with the RP-HPLC method. That disparity was consistent with degradation products either not eluting from the column or not being integrated. In order to investigate whether products were not eluting from the column, the gradient RP-HPLC method was modified to ramp to a very high organic content (90% acetonitrile) and held at this high organic content for 1 hour. This did not result in the elution of any additional degradation products, suggesting that there were not any highly retained, nonpolar degradation products. To test the possibility of poorly chromatographing degradation products, another experiment was conducted. In this experiment, the sample was assayed using an isocratic HPLC method with a high organic content to elute the parent drug and the degradation products as a single peak. The result obtained was comparable to the result from the flow injection analysis, suggesting that all degradation products were being eluted from the column. That finding suggested that poorly chromatographing degradation products were not being integrated with the original RP-HPLC method. This hypothesis was confirmed by modifying the original RP-HPLC gradient to a steep gradient (0–90% acetonitrile over 25 min). The resulting chromatograms, comparing unstressed samples and degraded stressed samples (with poor mass balance), are shown in Figure 4. Note the elevated baseline in the region of approximately 3–8 min that is seen only with the stressed sample and not with the control sample. Integration of this baseline “hump” and inclusion in the total peak area (parent and total impurities) provided results that were similar to those obtained using flow injection analysis. This baseline hump could also be recognized in samples that were not as extensively degraded when the chromatograms were overlaid with unstressed and blank sample chromatograms (as shown in Fig. 5). Thus, the low mass balance was attributed to poorly chromatographing species that were not previously integrated because they were disregarded as mere fluctuations in baseline.

Inaccurate Quantification Due to Differences in Response Factors

The importance of response factors to mass balance issues is such that a separate section of this chapter (see section “Practical Approaches to Solving Response Factor Problems”) is devoted to a more comprehensive discussion of this topic.

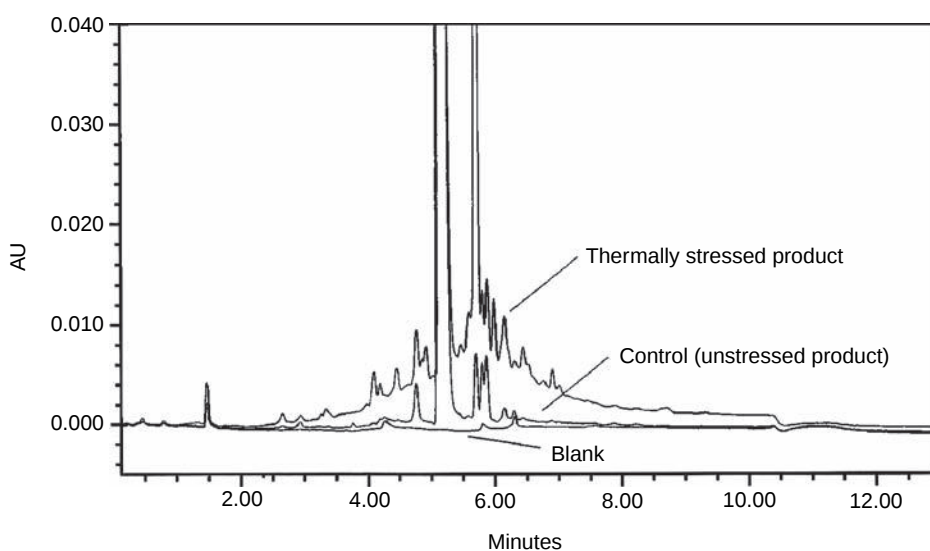


Figure 4 Overlaid HPLC chromatograms obtained on a thermally stressed drug product, a control (unstressed sample), and a blank using a steep-gradient HPLC method.

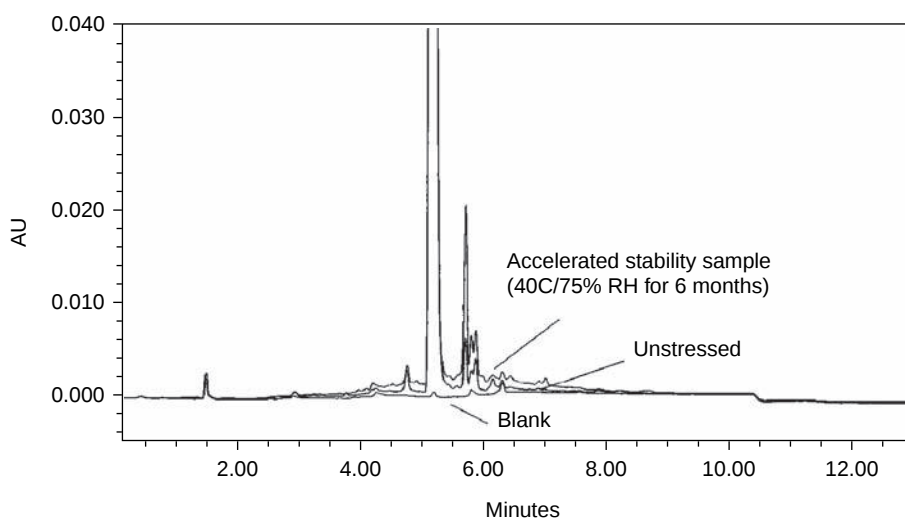


Figure 5 Overlaid HPLC chromatograms obtained on a drug product sample exposed to 6 months of accelerated stability conditions, an unstressed drug product sample, and a blank, analyzed using a steep-gradient HPLC method.

Negative Mass Balance Deficit

In these cases, the increase in mass (or number of moles) of degradation products is greater than the corresponding decrease in parent. Potential sources of the problem are described below.

- Inaccurate quantification of degradation product(s) due to differences in response factors. See section "Practical Approaches to Solving Response Factor Problems."
- Unaccounted-for reactants involved in addition reaction(s) to the parent compound. Consider reactants from matrix (oxygen, excipients, and container ingredients). Compare molar

mass balance to weight mass balance, that is, molar mass balance may be more appropriate in this case.

- Impurities arising from source(s) other than degradation of the parent compound. Such impurities could be present in the mobile phase, sample solvent, or column or could be from the sample matrix. Run a blank sample matrix, or one with varying concentrations of parent under the same conditions, to determine which—if any—impurities are unrelated to amount of parent present.
- Degradation product(s) co-eluting with the parent compound in the parent compound assay. Check for peak homogeneity via PDA-UV, LC-MS, and/or use an orthogonal separation technique, as discussed earlier. Modify conditions to separate if necessary.

PRACTICAL APPROACHES TO SOLVING RESPONSE FACTOR PROBLEMS

Poor mass balance is inevitable if response factors (e.g., absorptivity at the given wavelength) differ significantly between impurities and the parent compound when uncorrected peak areas are assumed to represent actual relative amounts (10). A positive mass balance deficit results if the response factors of the degradation products are less than that of the parent. A negative mass balance deficit results when the degradation products have larger response factors. Thus, relative response factors (RRFs) of impurities are an important consideration in assessing mass balance.

Occasionally, the UV response factors can be reasonably assumed to be quite similar; for example, if the parent and degradation product(s) share the same chromophoric backbone, with structural differences only in regions unassociated with the chromophore. Favorable comparison of UV spectra (e.g., from a PDA detector) can help confirm such a situation (26).

More often, structures are unknown or differ significantly in the chromophoric region of the molecule such that the assumption of similar response factors is questionable at best. Traditionally, the process for establishing response factors involves the use of isolated samples of individual impurities. For accurate determinations, the purity of each such sample must be known. For synthetic samples, the purity is generally estimated using a combination of HPLC (with UV, light-scattering, or other appropriate detection), NMR, and some method to determine volatile impurities (e.g., TGA and Karl-Fischer). This process involves a significant amount of time and effort (i.e., expense). The effort required is typically even greater for impurities that are not readily synthesized (e.g., low-level process impurities and degradation products). Such impurities need to be isolated and purified using standard techniques such as preparative TLC or HPLC. The risk of having contaminants in the isolated impurities is magnified when compared with synthetic samples because of the large amounts of solvents used, the possibility of nonchromophoric components (e.g., solvents or column bleed), the presence of counterions (e.g., trifluoroacetic acid, acetate, etc.), and the impracticality of using crystallization (with the low levels isolated) to enhance the purity. Moreover, in some cases, the degradation products are unstable and thus very difficult to purify. In order to assess sample purity, amounts of 50 mg or more are often needed. Isolation of these amounts of impurities can be very time consuming and costly. Once material of known purity is available, the determination of the response factor(s) is straightforward. A measured concentration is prepared and processed as per the analytical method, to determine the detector response (under the given conditions) per unit weight or molar concentration.

A potential alternative for determining UV response factors is to use two detectors: a standard UV absorbance detector and a second detector that has a response uniformly proportional to weight or concentration. For example, if a detector could provide accurate information on the relative amounts of the impurities and parent compound, then this information, combined with the UV peak areas, would supply the desired RRF information without the need for a purified impurity sample. One could reasonably question the need for a UV detector and RRF values at all if such an alternative detector was available, as it would directly provide information on relative amounts of impurities/parent. However, UV detectors are inexpensive, rugged, and readily available; therefore, RRF values, once determined, are widely applicable to situations in which no other detector is available.

While there is no truly "universal" HPLC detector, there are several detection methods that are more broadly applicable than UV absorption. These include refractive index (RI), electrical conductivity (EC), evaporative light scattering detection (ELSD), charged aerosol detection (CAD), chemiluminescent nitrogen detection (CLND), mass spectrometry (MS), and nuclear magnetic resonance (NMR). Each has its own strengths and weaknesses for assessment of mass balance or relative response factors, and will be discussed further below. There are also other, less common detection methods being researched that may prove quite valuable in the future [e.g., surface plasmon resonance (27), inductively coupled plasma atomic emission (28), and condensation nucleation light scattering (29,30)].

Mass spectral (MS) detectors are virtually universal, but the response per unit weight depends greatly on the type of ionization (e.g., electrospray, APCI, fast-atom bombardment, positive or negative ionization, etc.) and on the ionization efficiency of the analyte. Thus, except in specific cases where the responses are uniform, mass spectrometry is not an ideal detector for determining quantitative relationships between compounds based on peak areas (21–23).

¹H-NMR is one of the most general detectors for hydrogen-containing small molecules typical of pharmaceutical compounds. Quantitative NMR (qNMR) has recently been reviewed (31,32) and can be used in an off-line fashion with HPLC to determine RRFs. This methodology is particularly useful for situations where the species of interest is not available in the quantities typically needed for traditional HPLC-UV RRF determination. NMR provides an added advantage in that the purity of the compound of interest does not need to be known beforehand. An example of this approach has been thoroughly described elsewhere (33); a slight variation to this methodology will be summarized here.

The approach requires sufficient available impurity to obtain a quantitative ¹H-NMR spectrum: usually greater than 10 µg. This may necessitate chromatographic isolation prior to NMR analysis, or the level of the impurity might be high enough that an NMR spectrum can be obtained in the presence of the parent drug without the need for isolation. The NMR spectrum of the impurity is compared to a spectrum of parent drug in the same solvent, in which both compounds are fully soluble. At least one NMR resonance peak specific to each compound is then selected, such that the peaks are fully resolved and readily integrated. Ideally, the hydrogen responsible for each peak is already assigned from a known structure; however, it is sufficient to know only the molecular weight and the number of protons associated with each peak. An NMR spectrum is obtained of an approximately equimolar mixture (in the ideal case) of the impurity and the parent drug, and the areas are determined for the selected peaks (e.g., Fig. 6). Each area is normalized by dividing by the number of protons. The ratio of these normalized peak areas (impurity/parent) is equivalent to the molar ratio of the impurity to the parent. Some of the solution from the NMR tube is removed and diluted in HPLC mobile phase. This diluted sample is then injected on the HPLC-UV using a method that resolves the impurity from the parent (Fig. 7). It is important to remain in the linear region of the UV detector. The chromatographic peaks are integrated and the peak area ratio at the given wavelength is obtained. Using the parent drug rather than another standard (33) has the benefit that the parent serves as the internal standard and that the chromatographic method for the parent can be used. Finally, the RRF is calculated using the HPLC-UV and NMR peak area ratios, the number of protons, and the molecular weight of the two species [see Eq. (1)].

$$\begin{aligned} \text{RRF}_\lambda &= \frac{[(\text{UV Peak Area})/(\text{MW} \times (\text{NMR Peak Area}/\#H))_i]}{[(\text{UV Peak Area})/(\text{MW} \times (\text{NMR Peak Area}/\#H))_p]} \\ &= \frac{[(\text{UV Peak Area})_i \times \#H_i \times (\text{NMR Peak Area})_p \times \text{MW}_p]}{[(\text{UV Peak Area})_p \times \#H_p \times (\text{NMR Peak Area})_i \times \text{MW}_i]} \end{aligned} \quad (1)$$

where *i* = impurity, *p* = parent compound; #*H* is the number of hydrogen associated with the given NMR peak, and MW is the molecular weight.

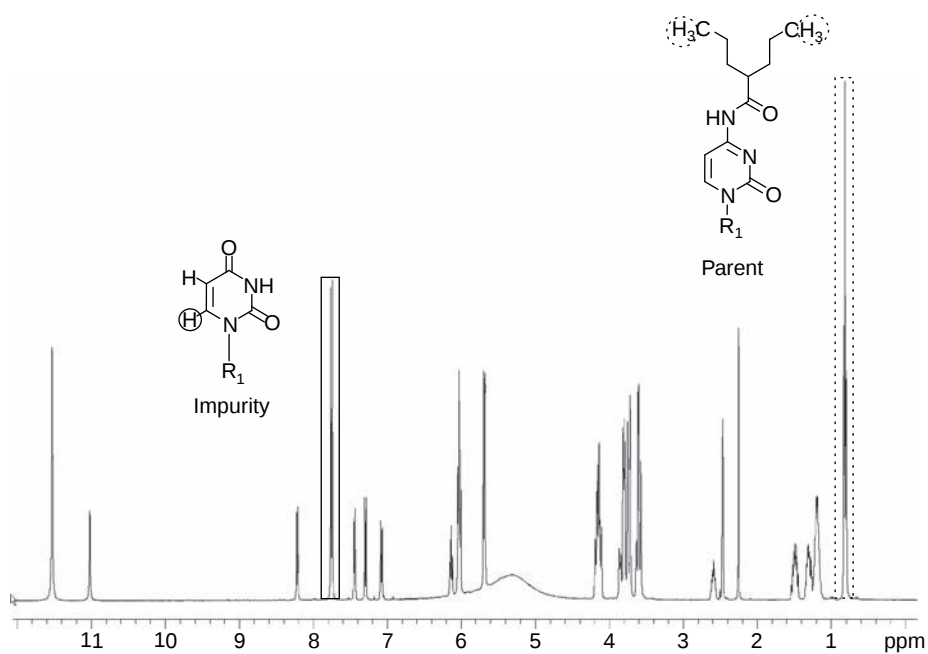


Figure 6 Specific ^1H -NMR peaks selected for parent compound and impurity (highlighted).

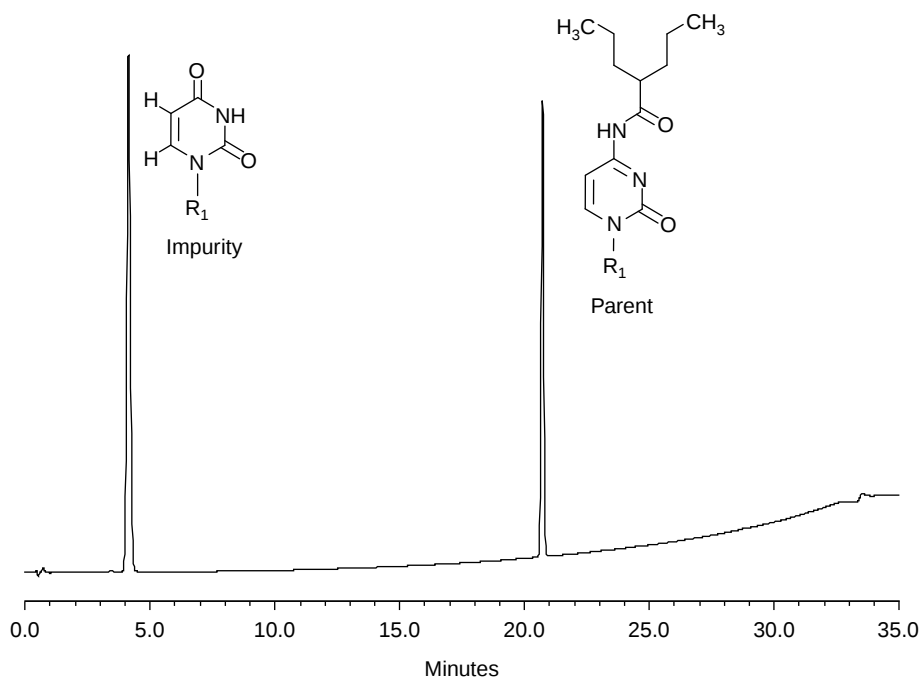


Figure 7 HPLC-UV chromatogram of parent compound and impurity at same relative concentrations as in Figure 6.

Measurement of changes in refractive index (RI) is one of the most universal detection strategies, in that virtually any dissolved compound will cause the RI of the solvent to change. However, RI currently suffers from variability in response depending on the mobile phase composition (e.g., gradients), temperature, and dissolved gases; furthermore, it has historically been relatively insensitive (34,35). Recently, though, improvements in RI detection sensitivity, gradient compatibility, and temperature stability have been reported (35). If such improvements become widely available and applicable to routine HPLC methods, RI will likely be much more broadly utilized for mass balance and RRF determinations in the future.

Electrical conductivity (EC) is widely used in ion chromatography. It is a particularly useful detection mode for charged species, but may also be used in an indirect mode for uncharged analytes. It is not uniformly sensitive to all substances, however. Also, EC is highly dependent on the mobile phase composition and so has historically been poorly suited to gradient elution (34). The addition of mobile phase ion-suppression typically converts the mobile phase primarily to pH 7.0 water, thereby reducing background and improving gradient compatibility of EC. The pH adjustment associated with ion-suppression does, however, require consideration of analyte pK_a and associated ionization state at the final pH if EC is to be used. Finally, it should be noted that suppression also reduces UV background since water is one of the most transparent UV solvents. Thus, ion suppression has significantly strengthened the ability to measure UV responses, in addition to EC, down to detection wavelengths as low as 190 nm (36,37).

Evaporative light-scattering detection (ELSD) has proven to be a widely applicable detector for nonchromophoric compounds. It is inexpensive, relatively rugged, easy to use and can be incorporated in many HPLC methods, although it is limited to volatile mobile phases. For compounds of similar structures and vapor pressures, one can expect similar responses per unit mass by ELSD (± 10 –20%) (38). Under such circumstances, the ELSD can be used in conjunction with a UV detector to determine RRF values by peak area ratios:

$$RRF_{\lambda} = \frac{[(UV \text{ Peak Area})/(ELSD \text{ Peak Area})]_i}{[(UV \text{ Peak Area})/(ELSD \text{ Peak Area})]_p} \quad (2)$$

where i = impurity, p = parent compound.

The assumption for Eq. (2) is that the ELSD peak area is directly proportional to mass. However, the ELSD response depends on the quantity and nature of the particles produced upon drying. For compounds of widely varying structures, charges, or vapor pressure, or for varying mobile phase compositions (e.g., gradient HPLC), the ELSD response can vary markedly. Thus, Eq. (2) is limited in its applicability. Finally, the ELSD signal is not linear with mass of analyte, so calibration can be more complex than with UV absorbance detectors (34,38–43).

A relatively recent detection method, charged aerosol detection (CAD), is similar to ELSD in converting the eluting analyte to fine particles. However, rather than using light scattering, CAD applies a charge to the particles via a corona discharge and measures the quantity of charged particles by means of an electrometer. As a result, CAD is more sensitive than ELSD for most nonvolatile analytes and has been reported to have a wider dynamic range (19,44–47). The CAD response is not quite linear, but is rather a quadratic fit, with respect to mass of analyte (48,49). Also, the response is primarily dependent on the particle size, and not on the structure of the analyte, provided that it is nonvolatile. The CAD signal is less dependent than ELSD on the nature (e.g., crystallinity and solvation) of the particles and so gives more uniform response factors for a wide variety of nonvolatile components. CAD is as rugged and easy to use as ELSD and has shown significant potential for determinations of relative UV response factors for pharmaceutical degradation products (19,45). However, the analytes must have virtually no vapor pressure under the conditions used in order to give consistent responses per unit mass. Also, changes in mobile phase composition promote changes in aerosol droplet

formation and therefore cause changes in CAD response, much as is the case with ELSD. In general, the CAD signal for a given analyte increases as the organic (vs. aqueous) proportion of the mobile phase increases. Thus, gradient reversed-phase HPLC-CAD tends to accentuate the later eluting peaks relative to those that elute earlier. Peak areas across a gradient cannot, therefore, be assumed to directly correlate to relative masses or molar amounts (19,45–49). However, it is possible to eliminate this error by mixing the eluent 1:1 with eluent from a second, opposite gradient prior to reaching the CAD. In this way, the organic/aqueous ratio is held constant at the CAD throughout the run (49).

A useful detector for RRF determinations of nitrogen-containing compounds is the chemiluminescent nitrogen-specific HPLC detector (CLND). This detector is based on combustion of the HPLC effluent in an oxygen-rich furnace to convert all organic species to oxides of carbon, nitrogen, sulfur, etc., and water. The nitric oxide produced from nitrogen-containing compounds is then reacted with ozone to produce nitrogen dioxide in an excited state, which emits photons upon return to the ground state (Fig. 8 for a schematic of the instrument). This chemiluminescent response is proportional to the number of moles of nitric oxide and correspondingly to the number of moles of nitrogen originally present in the analyte. For virtually any nitrogen-containing compound (with the exception of N_2 and compounds containing $N=N$ bonds), the signal is independent of structure. Thus, amines, amides, nitrates, nitrogen-containing heterocycles, etc., all produce a signal directly related to the number of moles of nitrogen present. Provided that the molecular formula of the analyte is known, one can thereby determine its relative weight in the sample from the amount of nitrogen in the HPLC peak.

Quantification, then, requires only a single nitrogen-containing standard, which need not be structurally related to the analyte. Thus, the CLND opens a new avenue for concentration determinations in the absence of standards of the given analyte (50–54). Moreover, for determination of relative amounts, no standard whatsoever is necessary. All that are needed are the relative CLND peak areas and the molecular formulas of the analytes. Once the relative amounts are found, it is a simple matter to use UV peak areas (e.g., from a UV detector in series with the CLND) to determine the RRFs. Thus, UV response factors (per unit weight) for impurities relative to parent compounds can be determined by means of the following equation:

$$RRF_{\lambda} = \frac{[(UV \text{ Peak Area}) / (CLND \text{ Peak Area})]_i}{[(UV \text{ Peak Area}) / (CLND \text{ Peak Area})]_p} \times \frac{(MW / \#N)_p}{(MW / \#N)_i} \quad (3)$$

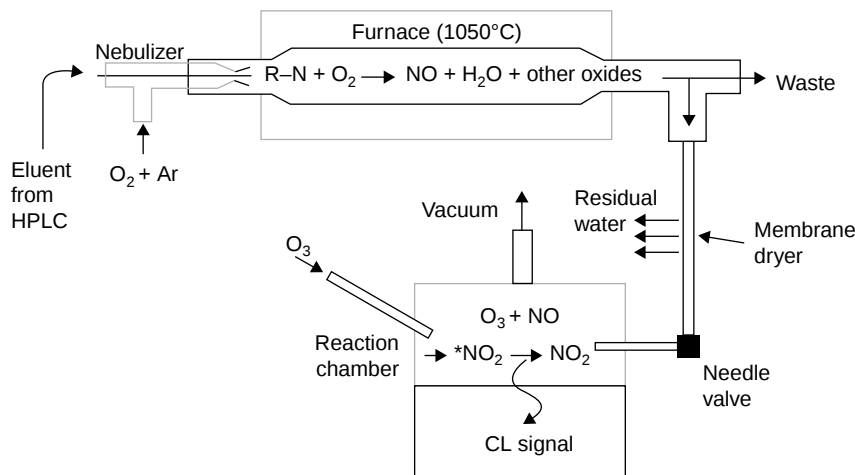


Figure 8 Schematic of CLND instrumentation.

where i is the impurity, p is the parent compound, MW is the molecular weight., and $\#N$ is the number of nitrogen in the molecular formula. Note that for unknown impurities, high resolution LC-MS can be used to determine the molecular formula (55–57). For molar (rather than weight) RRF values, one needs only the relative number of nitrogen per molecule and not the molecular weight.

As a result of the equimolar nature of the CLND, then, RRF information can be obtained without fraction collection or purification, without standards, and without even knowing the concentration of analyte. As a result, sample preparation is greatly simplified, and the stability of the impurity is not an issue.

The CLND is limited, of course, to mobile phases that do not contain nitrogen. Acetonitrile and amine modifiers, commonly used in HPLC, are therefore precluded. In addition, the CLND is not readily amenable to nonvolatile buffers in the mobile phase. However, it is still possible to determine RRF values for samples run under these non-CLND-compatible HPLC conditions. In such cases, a two-step process is used. First, a CLND-compatible mobile phase (e.g., methanol/water/trifluoroacetic acid) is used to separate the compounds of interest and determine RRF values under those conditions (RRF_1). Separately, the UV peak areas obtained using both the CLND-compatible and noncompatible HPLC conditions are compared by analyzing a common sample by both sets of HPLC conditions (apart from the CLND). The peaks of interest must, of course, be tracked to avoid misassignment (e.g., through UV spectra comparison). The relative response factor (RRF_1) obtained for the CLND-compatible method can then be used to determine the relative response factor (RRF_2) for a different set of conditions by multiplying by the ratio of the relative UV areas obtained under each:

$$RRF_2 = RRF_1 \times \frac{(\text{UV Peak Area})_{i,2}/(\text{UV Peak Area})_{i,1}}{(\text{UV Peak Area})_{p,2}/(\text{UV Peak Area})_{p,1}} \quad (4)$$

where i is the impurity, p is the parent compound, and 1 and 2 represent CLND-compatible and noncompatible HPLC conditions, respectively.

Alternatively, one can analyze a sample with the CLND to determine the relative amounts of the analytes present. Then, the identical sample can be analyzed with UV detection using non-CLND-compatible HPLC conditions, and the RRF calculated using Eq. (3). This approach requires that the non-CLND-compatible HPLC conditions be defined and run at the time of the CLND analysis, using the same sample. By contrast, the approach described with Eq. (4) permits running a separate sample at any time at a site removed from the CLND, albeit that sample must be run with UV detection under both sets of HPLC conditions.

RESPONSE FACTOR EXAMPLE: NIFEDIPINE

As a simple example of a positive mass balance deficit caused by different response factors, and corrected by use of the CLND, consider the photodegradation of nifedipine. Nifedipine (4-(2'-nitrophenyl)-2,6-dimethyl-3,5-dimethoxycarbonyl-1,4-dihydropyridine) is a well-characterized light-sensitive pharmaceutical compound. Upon exposure to sunlight or even room light, it rapidly oxidizes in solution to form 4-(2'-nitrosophenyl)-pyridine (see structures in Fig. 9) (58). This degradation product has a significantly different UV absorption spectrum than nifedipine. As a result, the RRFs of the two compounds are dissimilar at most wavelengths. Thus, mass balance by HPLC–UV is unlikely unless these RRF values are taken into consideration. Use of the CLND allows one to determine both the true mass balance and the RRF values, as described below (54).

A sample of nifedipine with minimal exposure to light was analyzed at its λ_{max} (237 nm) in order to get a precise measure of the CLND and UV peak areas for the parent compound. The sample solution was then degraded by exposing to a projector lamp over the course of 6 hours.

Aliquots were analyzed regularly throughout this time period by HPLC–UV–CLND. After 6 hours of degradation, the lamp was turned off and the sample was analyzed at this final time point. Sample chromatograms show the progress of conversion to the oxidation product over time (Fig. 10). Each peak was integrated with both UV and CLND detectors.

With UV detection at 237 nm, the total peak area (i.e., sum of the peak areas of nifedipine and its oxidation product) decreased throughout the photodegradation of the nifedipine sample. For example, the total UV peak area after 6 hours was only 65% of its initial value, suggesting a mass balance deficit. However, the total peak area by CLND was consistent throughout, with an overall RSD of 2.5%. Thus, the CLND indicated that the decrease in total UV peak area was due to different RRF values for the two compounds. Furthermore, the RRF value for the oxidation product was readily calculated using Eq. (3): $\text{RRF}_{237\text{ nm}} = 0.534$ on a weight basis.

Omitting the molecular weight information gives the RRF value on a molar basis: 0.563. By using this RRF value, the UV results were readily corrected. Finally, the appropriate RRF_2 for

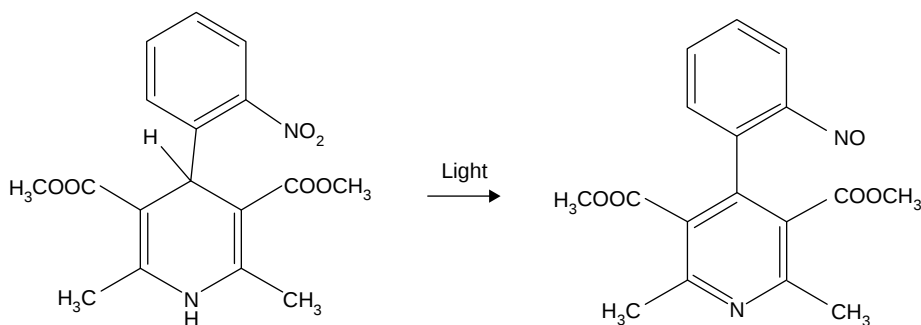


Figure 9 Photodegradation of nifedipine (58).

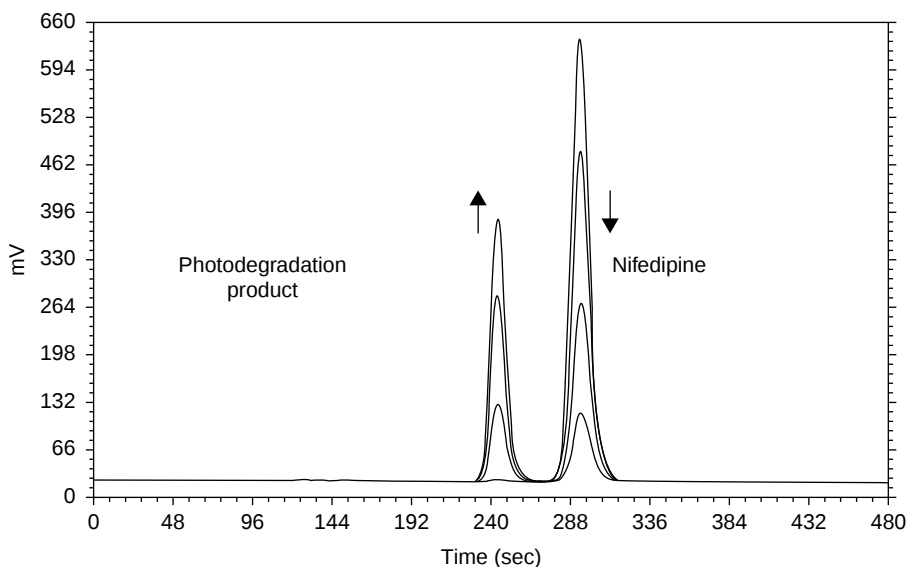


Figure 10 Photodegradation of nifedipine as monitored by HPLC/UV. HPLC conditions: Zorbax Rx-C18, 0.21×15 cm; mobile phase: 60/40 MeOH/H₂O, 0.2 mL/min; 5 mL inj. Detection: UV = 237 nm. Arrows indicate the progression of peak areas with time.

Table 1 Mass Balance of Photodegraded Nifedipine: Before and After RRF Correction from CLND Data

Sample	Nifedipine Peak Area	Ox. Product Raw Peak Area	Total Peak Area	AMBD (Area Units)	RMBD	Ox. Product Corrected Area	Total Peak Area	AMBD (Area Units)	RMBD
Initial	13658	105	13763	n/a	n/a	168	13826	n/a	n/a
Partially degraded	7558	4070	11628	2135	35%	6491	14049	-223	-3.7%
Fully degraded	16	8977	8993	4770	35%	14318	14334	-508	-3.7%

non-CLND-compatible conditions can be easily determined and broadly applied (e.g., in other laboratories). For example, the USP monograph assay for nifedipine uses a mobile phase of water:acetonitrile:methanol (50:25:25). By running a partially degraded sample via HPLC–UV under both sets of conditions, the molar RRF₂ value for the USP monograph conditions was calculated from Eq. (3) to be 0.627. Thus, peak areas obtained for the oxidation product using these conditions were corrected by dividing by 0.627. As a test case, initial, partially, and fully degraded nifedipine samples were run under the USP HPLC conditions. The corrected and uncorrected results are shown in Table 1. The AMBD and RMBD values were calculated as shown in the earlier definitions.

Note from Table 1 that, without RRF correction, the apparent mass balance is quite poor, with a consistent RMBD of 35%. Thus, in the absence of RRF information, one might conclude that mass balance was not achieved and that other, undetected degradation products were being formed or that parent compound was being lost (e.g., via adsorption and volatilization). Use of the RRF₂ value mentioned earlier, however, indicates excellent mass balance, even for a fully degraded sample (RMBD = -3.7%).

CONCLUSIONS

Mass balance is an important consideration in assessing degradation pathways of pharmaceutical products. Absolute and relative mass balance deficits are useful means of expressing deviations from true mass balance. Many of the most common sources of mass balance discrepancies and approaches to resolving them have been discussed. Often, response factor differences between degradation products and the parent compound are responsible for mass balance problems. Relative response factors (RRFs) should therefore be incorporated, when possible, in the quantification of degraded samples. Such response factor information can be obtained from purified samples of degradation products or by the use of specialized detectors such as charged aerosol detection or evaporative light scattering detection (for compounds of similar structure and vapor pressure), NMR, or chemiluminescent nitrogen-specific detection (for most nitrogen-containing compounds). Alternatively, a lack of detection (e.g., from loss of chromophore with UV detection or from long elution times on HPLC) may be encountered. In these cases, the use of different detectors and alternate separation techniques can help to uncover the source of the problem. By incorporating the concepts and techniques outlined in this chapter, the analytical chemist can begin to assess whether mass balance issues are truly indicating a need to further elucidate degradation pathway information.

ACKNOWLEDGMENTS

The contributions of Bernd Riebeschl and Heiko Brunner to the development of the concepts of absolute mass balance deficit and relative mass balance deficit are gratefully acknowledged.

REFERENCES

1. Kirschbaum JJ. Synergistic use of multiple assays and achievement of mass balance to validate analytical methods. *Trends Anal Chem* 1988; 7: 16–20.
2. International Conference on Harmonization Tripartite Guideline: Stability Testing of New Drug Substances and Products, ICH Q1A(R2), November, 2003.
3. Baertschi, SW. Analytical methodologies for discovering and profiling degradation-related impurities. *Trends Anal Chem* 2006; 25: 758–67.
4. Alsante KM, Ando A, Brown R, Ensing J, Hatajik TD, Kong W, Tsuda Y. The role of degradant profiling in active pharmaceutical ingredients and drug products. *Adv Drug Deliv Rev* 2007; 59: 29–37.
5. Conner KA, Amidon GL, Stella VJ. In: *Chemical Stability of Pharmaceuticals—A Handbook for Pharmacists*. 2nd edn. New York: Wiley, 1986.
6. Riley CM, Rosanske TW, eds. Development and validation of analytical methods. *Prog Pharm Biomed Anal* 1996; 3.
7. Priestner AA. Use of radiolabeled drug substance to investigate mass balance during validation of a high-performance liquid chromatography method for impurities. *Anal Proc* 1993; 30: 374–7.
8. Bakshi M, Singh S. ICH guidance in practice: establishment of inherent stability of secnidazole and development of a validated stability-indicating high-performance liquid chromatographic assay method. *J Pharm Biomed Anal* 2004; 36: 769–75.
9. Shabir, GA. Validation of high-performance liquid chromatography methods for pharmaceutical analysis: understanding the differences and similarities between validation requirements of the US Food and Drug Administration, the US Pharmacopeia and the International Conference on Harmonization. *J Chromatogr A* 2003; 987: 57–66.
10. Newton MP, Mascho J, Maddux RJ. Chromatography of pharmaceuticals: natural, synthetic, and recombinant products. In: Ahuja S, ed. *Chromatography of Pharmaceuticals*. ACS Symposium Series 512. New York: ACS Publications, 1991.
11. Olsen BA, Argentine MD. Investigation of response factor ruggedness for the determination of drug impurities using high-performance liquid chromatography with ultraviolet detection. *J Chromatogr A* 1997; 762: 227–33.
12. Guillemin CL, Gressin JC, Caude MC. The deferred standard method—a technique for quantitative analysis in laboratory and process HPLC. *J High Res Chrom Chrom Com* 1982; 5: 128–33.
13. Oyler AR, Armstrong BL, Dunphy R, Alquier L, Maryanoff CA, Cohen JH, Merciadiez M, Khublall A, Mehta R, Patel A, Il'ichev Y. Mass balance in rapamycin autoxidation. *J Pharm Biomed Anal* 2008; 48: 1368–74.
14. McCrossen SD, Bryant DK, Cook BR, Richards JJ. Comparison of LC detection methods in the investigation of non-UV detectable organic impurities in a drug substance. *J Pharm Biomed Anal* 1998; 17: 455–71.
15. Olsen, BA, and Baertschi, SW. Strategies for investigation and control of process-related and degradation-related impurities in pharmaceuticals. In Ahuja, S., and Alsante, K.M., eds. *Handbook of Isolation and Characterization of Impurities in Pharmaceuticals*, volume 5 of *Separation Science and Technology*, New York: Academic Press, 2003.
16. International Conference on Harmonization Tripartite Guideline: Stability Testing of New Drug Substances and Products, ICH Q1A, September, 1994.
17. Lane S, Boughtflower B, Mutton I, Paterson C, Farrant D, Taylor N, Blaxill Z, Carmody C, Borman P. Toward single-calibrant quantification in HPLC. A comparison of three detection strategies: evaporative light scattering, chemiluminescent nitrogen, and proton NMR. *Anal Chem* 2005; 77: 4354–65.
18. Vehovec T, Obreza, A. Review of operating principle and applications of the charged aerosol detector. *J Chromatogr A* 2010; 1217: 1549–56.
19. Gamache PH, McCarthy RS, Freeto SM, Asa DJ, Woodcock MJ, Laws K, Cole RO. HPLC analysis of nonvolatile analytes using charged aerosol detection. *LCGC North America* 2005; 23: 150–61.
20. Righezza M, Guiochon G. Effects of the nature of the solvent and solutes on the response of a light-scattering detector. *J Liq Chromatogr* 1988; 11: 1967–2004.
21. Cech NB, Krone JR, Enke CG. Predicting electrospray response from chromatographic retention time. *Anal Chem* 2001; 73: 208–13.
22. Tang L, Kobarle P. Dependence of ion intensity in electrospray mass spectrometry on the concentration of the analytes in the electrosprayed solution. *Anal Chem* 1993; 65: 3654–68.
23. Cheng ZL, Siu KW, Guevremont R, Bergman SS. Dependence of ion intensity in electrospray mass spectrometry on the concentration of the analytes in the electrosprayed solution. *J Am Soc Mass Spectrom* 1992; 3: 281–8.

24. Petritis K, Elfakir C, Dreux M. A comparative study of commercial liquid chromatographic detectors for the analysis of underivatized amino acids. *J Chrom A* 2002; 961: 9–21.
25. Baertschi SW. The role of stress testing in pharmaceutical product development (oral presentation). AAPS Midwest Regional Meeting, Chicago, IL, May 19, 1996.
26. Olsen BA, Baertschi SW, Riggins RM. Multidimensional evaluation of impurity profiles for generic cephalixin and cefaclor antibiotics. *J Chromatogr* 1993; 648: 165–73.
27. Du M, Zhou F. Postcolumn renewal of sensor surfaces for high-performance liquid chromatography – surface plasmon resonance detection. *Anal Chem* 2008; 80: 4225–30.
28. Paredes E, Maestre SE, Prats S, Todoli JL. Simultaneous determination of carbohydrates, carboxylic acids, alcohols, and metals in foods by high-performance liquid chromatography inductively coupled plasma atomic emission spectrometry. *Anal Chem* 2006; 78: 6774–82.
29. Allen L, Koropchak JA, Szostek B. Condensation nucleation light scattering detection for conventional reversed-phase liquid chromatography. *Anal Chem* 1995; 67: 659–66.
30. Koropchak JA, Sadain S, Yang X, Magnusson L, Heybroek M, Anisimov M. Fundamental aspects of aerosol-based light scattering for separations detection. *Advances Chromatogr* 2000; 40: 275–314.
31. Holzgrabe U, Deubner R, Schollmayer C, Waibel B. Quantitative NMR spectroscopy—applications in drug analysis. *J Pharm Biomed Anal* 2005; 38: 806–12.
32. Holzgrabe U. qNMR spectroscopy in drug analysis—a general view. In: Holzgrabe U, Wawer I, Diehl B, eds. *NMR Spectroscopy in Pharmaceutical Analysis*, Amsterdam; Elsevier, 2008
33. Webster GK, Marsden I, Pommerening C, Tyrakowski CM, Tobias B. Determination of relative response factors for chromatographic investigations using NMR spectrometry. *J Pharm Biomed Analysis* 2009; 49: 1261–5.
34. Snyder LR, Kirkland JJ, Glajch JL. *Practical HPLC Method Development*, 2nd edn. New York: Wiley, 1997: 80–1.
35. Wang Z, Bornhop DJ. Dual-capillary backscatter interferometry for high-sensitivity nanoliter-volume refractive index detection with density gradient compensation. *Anal Chem* 2005; 77: 7872–7.
36. Reed RA, Bowen B, Harmon P, Rittenhouse J, Rivera S, Tyrrell RJ Yin, W. The utilization of deep ultraviolet detection with ion suppression: a case study of the highly acidic amine, mechloroethamine, pre- and post- γ -irradiation. *International Ion Chromatography Symposium* 1995, Dallas, TX, October 2–5, 1995.
37. Cassidy SA, Demarest CW, Wright PB, Zimmerman B. Development and application of a universal method for quantitation of anionic constituents in active pharmaceutical ingredients during early development using suppressed conductivity ion chromatography. *J Pharm Biomed Anal* 2004; 34: 255–64.
38. Fang L, Wan M, Pennacchio M, Pan J. Evaluation of evaporative light-scattering detector for combinatorial library quantitation by reversed phase HPLC. *J Comb Chem* 2000; 2: 254–7.
39. Cebolla VL, Membrado L, Vela J, Ferrando AC. Understanding evaporative light scattering detection for high-performance liquid chromatography. *Sem Food Anal* 1997; 2: 171–89.
40. Hopia AI, Ollilainen VM. Comparison of the evaporative light scattering detector (ELSD) and refractive index detector (RID) in lipid analysis. *J Liq Chromatogr* 1993; 16: 2469–82.
41. McNabb TJ, Cremesti AE, Brown PR, Fischl AA. High-performance liquid chromatography/evaporative light-scattering detector techniques for neutral, polar, and acidic lipid classes: a review of methods and detector models. *Sem Food Anal* 1999; 4: 53–70.
42. Kibbey CE. Quantitation of combinatorial libraries of small organic molecules by normal-phase HPLC with evaporative light-scattering detection. *Mol Divers* 1995; 1: 247–58.
43. Hsu BH, Orton E, Tang S, Carlton RA. Application of evaporative light scattering detection to the characterization of combinatorial and parallel synthesis libraries for pharmaceutical drug discovery. *J Chromatogr B* 1999; 725: 103–12.
44. Dixon RW, Peterson, DS. Development and testing of a detection method for liquid chromatography based on aerosol charging. *Anal Chem* 2002; 74: 2930–7.
45. Sun P, Wang X, Alquier L, Maryanoff C. Determination of relative response factors of impurities in paclitaxel with high performance liquid chromatography equipped with ultraviolet and charged aerosol detectors. *J Chromatogr A* 2008; 1177: 87–91.
46. Ramos RG, Libong D, Rakotomanga M, Gaudin K, Loiseau PM, Chaminade P. Comparison between charged aerosol detection and light-scattering detection for the analysis of Leishmania membrane phospholipids. *J Chrom A* 2008; 1209: 88–94.

47. Vervoort N, Daemen D, Torok G. Performance evaluation of evaporative light scattering detection and charged aerosol detection in reversed phase liquid chromatography. *J Chrom A* 2008; 1189: 92–100.
48. Operating and Maintenance Manual, Corona CAD Detector, ESA Biosciences, Chelmsford, MA USA, 2009.
49. Gorecki T, Lynen F, Szucs R, Sandra P. Universal response in liquid chromatography using charged aerosol detection. *Anal Chem* 2006; 78: 3186–92.
50. Fitch WL, Szardenings AK, Fujinari EM. Chemiluminescent nitrogen detection for HPLC: an important new tool in organic analytical chemistry. *Tetrahedron Lett* 1997; 38: 1689–92.
51. Taylor EW, Qian MG, Dollinger GD. Simultaneous online characterization of small organic molecules derived from combinatorial libraries for identity, quantity, and purity by reversed-phase HPLC with chemiluminescent nitrogen, UV, and mass spectrometric detection. *Anal Chem* 1998; 70: 3339–47.
52. Koerner A. Uncovering deficiencies in mass balance using HPLC with chemiluminescence nitrogen-specific detection. *LCGC North America* 2002; 20: 364–73.
53. Liang X, Patel H, Young J, Shah P, Raglione. The practical application of implementing the equimolar response principle of chemiluminescent nitrogen detection in pharmaceutical analysis. *J Pharm Biomed Anal* 2008; 47: 723–30.
54. Nussbaum MA, Baertschi SW, Jansen PJ. Determination of relative UV response factors for HPLC by use of a chemiluminescent nitrogen-specific detector. *J Pharm Biomed Anal* 2002; 27: 983–93.
55. Haskins NJ, Eckers C, Organ AJ, Dunk MF, Winger BE. The use of electrospray ionization with Fourier transform ion cyclotron resonance mass spectrometry in the analysis of trace impurities in a drug substance. *Rapid Commun Mass Spectrom* 1995; 9: 1027–30.
56. Perkins G, Pullen F, Thompson C. Automated high resolution mass spectrometry for the synthetic chemist. *J Am Soc Mass Spectrom* 1999; 10: 546–51.
57. Palmer ME, Clench MR, Tetler LW, Little DR. Exact mass determination of narrow electrophoretic peaks using an orthogonal acceleration time-of-flight mass spectrometry. *Rapid Commun Mass Spectrom* 1999; 13: 256–63.
58. Pietta P, Rava A, Biondi P. High-performance liquid chromatography of nifedipine, its metabolites and photochemical degradation products. *J Chromatogr* 1981; 210: 516–21.

10 Solid-state pharmaceutical development: Ensuring stability through salt and polymorph screening

Susan M. Reutzel-Edens and Greg A. Stephenson

INTRODUCTION

Physical and chemical properties of an active pharmaceutical ingredient that affect the performance of solid oral dosage forms, such as chemical stability, mechanical properties, hygroscopicity, solubility and dissolution rate, are strongly influenced by the solid-state form of the drug substance. Since drug product performance can only be assured when a drug compound is delivered in a consistent manner to the patient, pharmaceutical solids must be both chemically and physically stable. Chemical stability is the resistance to chemical reactions, which give rise to covalent bond changes (i.e., breaking or forming) under conditions relating to humidity, temperature, and photoirradiation, while physical stability refers specifically to the stability of a solid form with respect to polymorph transformations, hydration or dehydration, salt disproportionation, crystallization, or amorphization. Solid form (salt and polymorph) screening to identify chemically and physically stable forms of a drug substance is an essential first step in pharmaceutical research and development. There are a number of excellent monographs, which independently cover aspects of salt and polymorph screening (1–3). The types of chemical reactions and physical transformations observed in bulk drug substances and formulations have also been thoroughly reviewed (4,5). The purpose of this chapter is to discuss the role of solid form screening and selection in ensuring the physicochemical stability of pharmaceuticals as drug substances and in solid oral drug products throughout their shelf life. Here, salt and polymorph screening are treated collectively as a key component of the product design. Although emphasis is clearly placed on identifying suitable crystalline forms of drug substances, the implications of amorphous forms introduced either by design or accident to ensure chemical and physical stability are also discussed.

PHARMACEUTICAL SOLIDS: AN OVERVIEW

Structural diversity in pharmaceutical solids arises from the conformational flexibility of most drug molecules and a repertoire of noncovalent interactions (hydrogen bonds, van der Waals, π -stacking, and electrostatic interactions), which hold molecules (and ions) at equilibrium in the solid state (6). While the different types of solid forms that may be encountered in drug development are generally recognized, the lack of universally accepted nomenclature and the lively debate in recent years over the use of solid-state terminology requires that at the very minimum, a basic description be provided to delineate how the terminology is used in this chapter (7). Solid forms may be differentiated by their chemical composition (number, identity, and stoichiometry of components), crystallinity, the ionization state of acidic/basic components (salts), and solid-state packing arrangements (polymorphs). The terms used to describe solid forms of drug compounds, along with many of the compositional and topological relationships between them, are highlighted in Figure 1. *Salt forms*, produced by acid–base reactions in the solid state, are multicomponent solids comprised minimally of a single A^-B^+ pair and may be crystalline or amorphous. The term *cocrystal*, on the other hand, specifically refers to multicomponent crystals, which by definition include solvates (hydrates), inclusion compounds, and clathrates, and may include an A^-B^+ pair among the components. Multicomponent amorphous solids, which are molecularly dispersed, are best described as solid dispersions. McCrone's widely accepted definition of *polymorph*: a solid crystalline phase of a given compound resulting from the possibility of a least two different arrangements of the molecules of that compound in the solid state, is used herein. The basic tenet of this definition is that the chemical composition of different polymorphs must be identical; just how different a crystal

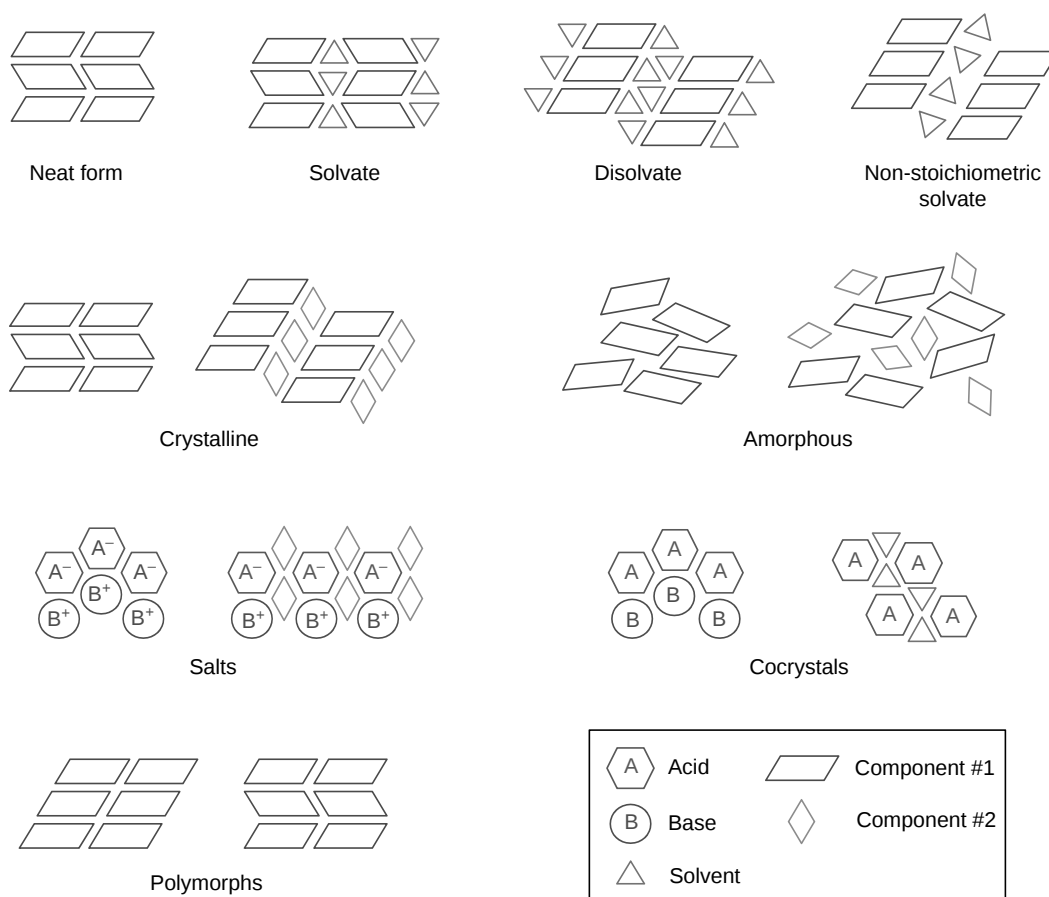


Figure 1 Structural diversity of pharmaceutical solids.

structure should be in order to be considered a different polymorph has been discussed elsewhere (8,9).

SOLID-STATE REACTIVITY

Crystalline forms of drugs are generally preferred to amorphous forms for many reasons. Among them are the ease of isolation, the high purity typically attained by crystallization, and the greater physical and chemical stability imparted by the crystalline state. Reactions generally occur more readily in solution and disordered solids than in well-ordered crystalline phases, wherein molecular mobility is reduced within a relatively rigid crystalline lattice. Paul and Curtin proposed a four step mechanism for thermally induced reactions in the solid state involving intramolecular change, uncomplicated by the necessity for reactants to diffuse together. Mechanistically, the first step involves molecular loosening at the reaction site, followed by a molecular change within the starting material and the formation of a solid solution, whereby product accumulates in the parent crystal until the “solubility” is exceeded and nucleation induces crystallization of a separate phase. The fourth step results in the separation of the product phase (10). More recently, Byrn, through omission of the step involving solid–solution formation, extended the mechanism to reactions that involve solvent-mediated transitions and those that require solvent vapor to proceed. In doing so, certain generalizations could be made about factors that influence the rate of solid–solid transitions involving solvents. Translocation

of molecules takes place such that temperature, viscosity, molecular size, and shape, as well as other diffusion-related factors play a role. The concentration of the intermediate phase will also affect the rate. Therefore, the absolute and relative solubilities of the solid phases in one another are important, as are impurities or specific solvent interactions that affect growth of the new solid phase (4). In reality, most reactions of greatest concern in pharmaceuticals are mediated by solvent vapors or liquid solvent, with that solvent generally being water. Polymorphic transformations are considered solid-state reactions, despite the lack of chemical transformation, and proceed by the described mechanisms.

Amorphous forms of pharmaceuticals are chemically and physically metastable with respect to crystalline phases due to their greater molecular mobility even in the absence of moisture. Pikal et al. have demonstrated that "rigorously dry," amorphous cephalosporin pharmaceuticals were at least one order of magnitude chemically less stable than their crystalline counterparts (11). With such a pronounced difference in reactivity in the dry state, one can appreciate the impact that even small levels of an amorphous component might have on the chemical stability of the solid. Similarly, trace levels of an amorphous component can enhance rates of physical transformations, such as crystallization and solid-state form conversions. In these instances, the activated state effectively reduces the energy barrier for nucleation of a more stable crystalline form.

Crystallinity does not guarantee that a drug substance will be chemically stable, since chemical degradation, including oxidation, cyclization, deamidation, hydrolysis, and the Maillard reaction, can also in principle occur in crystalline materials (12). In contrast to solution or gas phase reactions, solid-state reactions are severely constrained by limited molecular mobility within the confines of the crystalline lattice. Thus, while molecules in solution react more or less independently, a high level of cooperativity is required in a crystal lattice in order for the displacement of one molecule to occur. That disruption is communicated to its near neighbors and so on creating chemical pressure or localized stress fields (13,14). In rare instances, solid-state reactions proceed more efficiently and with greater selectivity than in solution because of the regular arrangement of tightly packed molecules. Prednisolone *tert*-butylacetate (PTBA) oxidation is a classic example where chemical reactivity is strongly impacted by the molecular arrangement in a crystal structure (15). The greater reactivity of PTBA Form V is attributed to the formation of tunnels parallel to the hexagonal sixfold screw axis in its crystal structure, which are penetrable by O₂ following the loss of water from the nonstoichiometric hydrate, Figure 2.

Topochemical reactions, wherein the structure of the product is dictated by the geometry and proximity of the reactive sites within the lattice, often occur at faster rates than solution-state reactions and in some instances impart complete stereospecificity (16,17). With the advent of area detectors for x-ray diffraction analysis, the mechanisms of such reactions have been studied in great detail, particularly for photochemical reactions that initiate internally within single crystals (18). Topochemically controlled photochemical reactions occur within the bulk of the crystals and represent solid-state reactivity in its purest sense with respect to the mechanism proposed by Paul and Curtin. The reacting atoms are generally within 4.2 Å of one another and there is mutual orientation of the reacting molecules within the lattice. Upon irradiation of the crystal, the reaction initiates with reactant-product disorder within the lattice being observed by x-ray diffraction. As the reaction progresses, the reactant molecule moves away from its initial position (molecular loosening) in the pure crystal as molecular reorientation occurs (molecular change), exerting stress on the parent crystal. The atoms of the product appear in slightly different positions from where they originally existed in the pure reactant crystal (solid solution formation) and subsequently move smoothly toward the position they assume in the pure product crystal (separation of the product). At minimum, this example serves to illustrate that solid-state reactions are dynamic in nature, with molecular mobility and cooperativity being required within the crystal lattice.

Few solid-state reactions are chemical transformations involving solely well-ordered crystalline phases. More commonly, reactions initiate at defect sites or amorphous domains

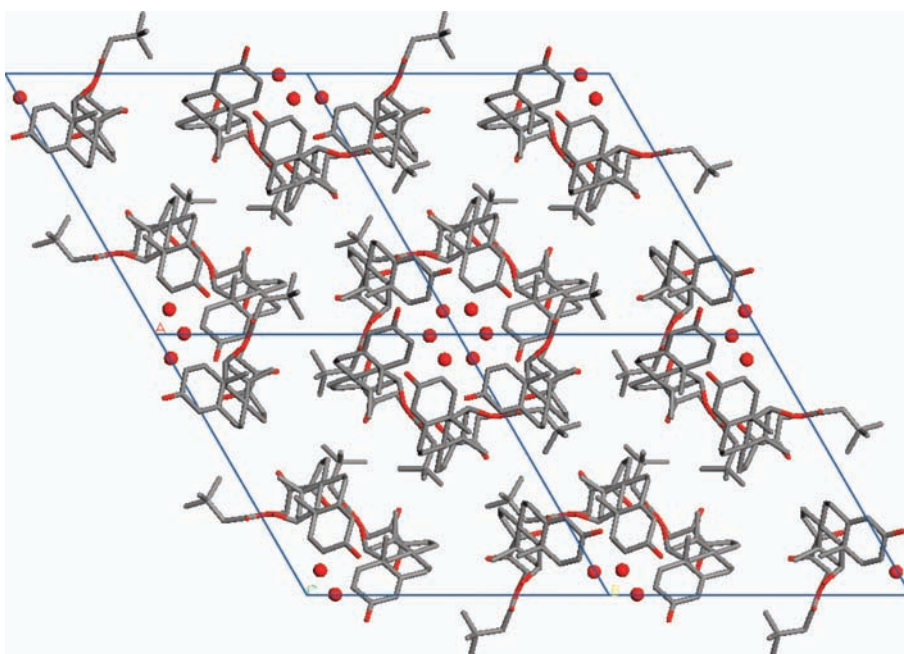


Figure 2 Crystal packing of prednisolone *tert*-butylacetate Form V. (CSD refcode: GAJMIN).

located on either the surface or within the bulk crystal. A well-studied example is the gas-solid reaction of indomethacin with ammonia (19). Indomethacin reacts in its metastable α -form with ammonia, while the thermodynamically stable γ -form is inert. In this case, the metastable α -form is *more* dense than the stable γ -form (contrary to the density rule). Because ammonia gas cannot diffuse into the crystal structure, molecular loosening is required. It is proposed that for indomethacin, the reaction (accessibility to the reactive site) of the α -form with ammonia initiates not only at disordered regions, but also at surfaces that expose the reactive functional groups, and then proceeds by diffusion. Such surfaces were not identified in the thermodynamically stable γ -form.

Reaction kinetics in the solid state are complex and highly variable, depending on both the solid-state form of the reactants (amorphous, polymorphs, and solvated forms) and the defect density (number of potential nucleation sites). Attempts to model and interpret solid-state kinetics have led to numerous controversies due to variable activation energies, different methods of calculation, and kinetic compensation effects (20). In contrast to solution, where the Arrhenius equation relates the rate constant of a reaction to temperature through an activation energy and pre-exponential factor, the activation energies and pre-exponential factors are assumed to remain constant as a reaction progresses in the solid state. Attempts to apply similar equations to the solid state have shown that these kinetic parameters may vary with reaction progress due to product formation, defect formation and propagation, and intracrystalline strain. Further complicating solid-state kinetic modeling is the artifactual variation in activation energies due to method of experimental analysis, mass transfer, thermal gradients across a sample, self-heating, etc. Clearly, measurement of reaction rates in the solid state is very challenging relative to their solution state counterparts, even when experiments are rigorously controlled. As a result, kinetic data may often fit multiple mechanistic models, making it difficult to infer with certainty the actual mechanism for a solid-state reaction.

Most solid-state reaction kinetics are affected by atmospheric water, which functions as a reactant, a product or the reaction medium, enhancing molecular mobility at disordered sites.

Water absorption into localized regions of high energy or disorder in otherwise crystalline materials can mediate transformations of solid reactants by increasing molecular mobility and in some instances, creating a “microenvironmental pH” conducive to chemical reactivity (21). Although the total water content in the solid may be low, the effects of absorbed water are amplified because the water that is present is concentrated in the amorphous regions (22). In such cases, problems encountered with drug products, originating from the drug substance, excipients, or both, may not be readily attributable to water sorption. Reactions moderated or enhanced by water are, in many cases, identified as those accelerated with increasing relative humidity (RH). Increasing RH does not always translate into enhanced reactivity of water mediated transformations, however. In studying the solid-state kinetics of aspirin hydrolysis, Ball modeled the chemical reactivity from decomposition curves and determined that the activation energy for the hydrolysis reaction was actually *greater* at higher humidity than at lower humidity (23). Modeling of the reaction data indicated that nucleation and growth phenomena were rate controlling and examination of aspirin single crystal surfaces by SEM showed etch pits to support a nucleation and growth mechanism. The greater activation energy for aspirin hydrolysis at higher humidity was explained in terms of the surface heterogeneity of the aspirin crystals. At lower vapor pressures, the most active sites react first, leaving those with higher activation energies to react at higher vapor pressures.

Deliquescence occurs when a substance absorbs water vapor from the atmosphere, leading to dissolution of the solid. The critical relative humidity (RH_0) at which this occurs is characteristic of a specific form of a substance and is equivalent to the RH of a saturated solution of that physical form in water. Below RH_0 , a deliquescent solid will adsorb minimal amounts of moisture at the crystal surface and will not encounter liquid water. Under these conditions, moisture-mediated reactivity will only occur because of water uptake at crystal defect sites or amorphous regions (having a different, lower RH_0). Above RH_0 , dissolution in the sorbed moisture can generate the supersaturation necessary for nucleation and growth of crystals, as well as enhance chemical reactivity. Taylor recently reported the synergistic influence of self-originating impurities and water vapor on the degradation kinetics of ranitidine HCl (24). Pure samples showed a sigmoidal shaped degradation profile for all storage conditions studied with minimal moisture sorption observed during the lag time. Once degradation commenced, however, the samples started to absorb moisture, even at low partial pressures of water. Impurities in contact with the surface of the drug, in combination with water vapor, were found to promote dissolution, thereby enhancing reactivity (25). The induction period commonly observed in solid-state reactions was eliminated through two mechanisms, one being the formation of hygroscopic liquid degradants leading to an increase in the portion of ranitidine hydrochloride in solution and the other being deliquescence lowering in the presence of a deliquescent impurity which further promoted a solid-liquid transformation of the active (26).

OPTIMIZING PHYSICAL AND CHEMICAL STABILITY THROUGH SOLID-STATE FORM SCREENING

Drug product design generally begins with identifying, oftentimes through crystallization screening, solid forms in which to isolate and store the drug substance. When performed under different conditions, crystallization can yield different forms (polymorphs, solvates) with different sizes and morphologies, thus providing an opportunity to “engineer” particles to desired specifications. Crystal engineering, which entails designing crystalline molecular solids with the aim of tailoring specific physical or chemical properties, though rarely discussed in this context for pharmaceuticals, is in fact the primary objective of salt and polymorph screening programs. Indeed, through solid-state form screening lies the opportunity to

- identify means of purifying drug substances through crystallization;
- impart chemical stability through immobilization of molecules in the crystalline state;
- enhance bioavailability (solubility, dissolution rate) of poorly water soluble drugs;

- assure the manufacturability of the drug substance and the dosage form;
- ensure drug product safety and efficacy through consistent systemic delivery of drugs to patients.

Whether it is to identify means to purify drug substances or to ensure their chemical stability, solid form screens will inevitably be initiated to identify viable crystalline forms of amorphous or poorly crystalline compounds as they enter drug development. Even crystalline compounds will undergo crystallization screening early in development if, for example, the solubility is deemed insufficient to achieve the high exposures required for establishing drug toxicity. Fortunately, drug compounds, frequently possessing acidic or basic functionality and always capable of hydrogen bonding, can potentially form salts or cocrystals, providing numerous options beyond the parent molecule to engineer viable solid-state forms.

To meet the design requirements for the drug product, a solid form with acceptable physical and chemical properties must be selected from potentially numerous options discovered through crystallization screening. A solid form landscape comprised of crystalline and amorphous forms ranked in the order of their stability (free energy) has been constructed for a model basic drug to illustrate the form diversity that may be encountered in the solid form selection process. As shown in Figure 3, the basic compound, alone or in the presence of an acidic guest, may crystallize during a screen in many different forms or it may not crystallize at all. Of all of the solid form alternatives, amorphous forms represent the most energetic solid forms, which should in theory provide the greatest solubility advantage. Hancock and Parks showed that compared to the most stable crystalline form, amorphous forms were approximately 10 to 1600 times more soluble (26). Crystalline salts and cocrystals are comparatively closer in free energy to the crystalline forms of the parent molecule. Salt solubility can be higher or lower, in some cases by orders of magnitude, than that of the free base (or acid), depending on the solution pH. The aqueous solubility of cocrystals was recently shown to be 2 to 152 times higher than the parent drug substance, though cocrystals of lower solubility are also possible (27). By comparison, the free energy differences between polymorphs and/or hydrates are relatively small, with solubility ratios of two or less being most common (28). Consideration of the various forms comprising the solid form landscape

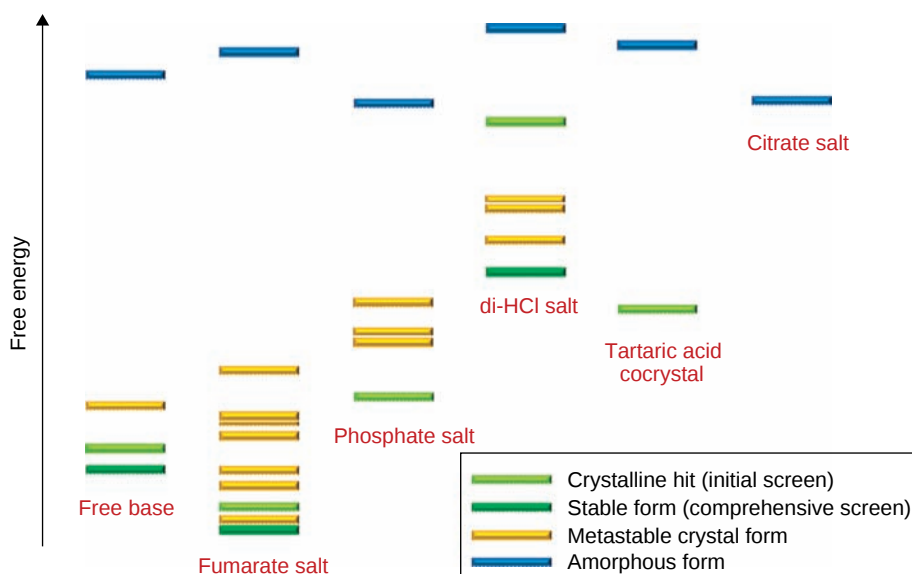


Figure 3 Solid-state form landscape of a basic drug compound at constant T , P .

and their free energy relationships provides a useful framework from which both a solid form may be selected to meet the design requirements of the drug product and the risks of physical instability that oftentimes lead to chemical instability may be understood. In the following sections, unique aspects of salt, cocrystal, polymorph and amorphous form screening needed to define the solid form landscape of a drug substance are examined.

Salts

Salt formation is perhaps the most commonly used technique for increasing the solubility and dissolution rate of an ever-increasing fraction of poorly soluble new chemical entities in drug product development. Indeed, an estimated 50% of all drug molecules are administered as salts (29). A recent analysis of the Orange Book published by the U.S. Food and Drug Administration (FDA) revealed the frequency with which different counterions have been used in approved drug products (30). The significantly greater frequency with which salts were used for injectable formulations suggested that the route of administration has influenced counterion selection. In this regard, salt formation appears to be more important for injectable products than oral formulations because considerably higher solubility (at least an order of magnitude) is required for injectables compared to oral formulations. Of course, salts can also be less soluble and dissolve more slowly than free acid or base forms. Less soluble forms have applications in parenteral depot drug products, slow-release oral dosage forms and inhalation products.

Counterion selection in salt screening begins with an assessment of the acidity or basicity of the ionizable functional groups on the drug molecule. A guiding principle for successful salt formation, the “rule of three”, is that the pK_a of the counterion should be about three units lower than the pK_a of a basic drug or three units higher than the pK_a of an acidic drug (31). Serajuddin and Pudipeddi have suggested that in search of acceptable salts, the salt screening process may be simplified and many unnecessary attempts to prepare salts may be eliminated by applying basic pH solubility principles, such as the rule of three (1). Strict application of the “rule of three” in salt screening should be tempered, however, by the understanding that combined errors involved in determination of the pK_a values of the reacting components can be significant. First, pK_a measurements are typically made in very dilute water solutions; however, for highly water insoluble compounds, multiple aqueous-organic solutions are typically used, from which the pK_a value in pure water is determined by extrapolation. In such cases, dissociation constants can be dramatically influenced by the dielectric constant of the solvent. For example, the measured pK_a values of carboxylic acids have been shown to shift higher by more than two units when the solution composition varied from 0% to 80% methanol (32). Generally, the conditions of crystallization differ substantially from the conditions in which pK_a values are measured and the solution in which the crystallization occurs is never dilute. Measured pK_a values commonly have experimental errors on the order of 0.5 pH units. Often salt screening is conducted before experimental pK_a values become available. In these cases, calculated pK_a values, which for heterocyclic and conjugated nitrogenous systems are frequently off by 1 to 2 pH units, are sometimes used to select counterions. It should be noted that while salt formation in solution may be predicted by pK_a differences, screening for crystalline salt forms remains a trial and error exercise; predictive structure-stability relationships have yet to be established for pharmaceutical salts (33).

The ability of acidic or basic drugs to form salts, as well as the ease with which they dissociate back to their free forms, depends not only on the pK_a difference (ΔpK_a) between the acid and base, but also on the intrinsic solubility (S_0) of the free form, the solubility product of the salt (K_{sp}) and the pH (34). Collectively, the pK_a , S_0 , and K_{sp} determine the pH-solubility profiles of a weak acid or base according to Eq. (1) (35).

$$pH = pK_a + \log \left(\frac{S_0}{K_{sp}^{0.5}} \right) \quad (1)$$

Figure 4 shows the pH solubility profile of a weak base ($pK_a = 5$). Application of the phase rule to the equilibrium between a salt and free base indicates that in a saturated solution at a given temperature and pressure, the addition of a small amount of an acid or base will not affect the pH or the concentration of the drug in solution until enough is added so that only a single phase remains. This pH, known as the pH_{max} , is the point at which maximum solubility occurs since both free base and salt forms of the molecule contribute to the total solubility of the drug in solution (36). Note that in the case of the weak base shown in Figure 4, the pH_{max} occurs at a pH significantly lower than the pK_a and its position on the pH-solubility profile depends on the salt form.

The concept of pH_{max} is fundamental to understanding the physical stability of the salt formed and its potential to dissociate back to its neutral form, commonly referred to as disproportionation. As shown in Figure 4, the more soluble the salt (e.g. HCl *vs.* tosylate), the lower the solution pH must be for the salt to be thermodynamically favored over its unionized form. While highly water soluble salt forms will be particularly susceptible to water-mediated phase transformations, the risk of disproportionation is not solely influenced by the solubility of the salt form. In a recent study of the disproportionation of miconazole and benzocaine salts, the *more* highly soluble miconazole mesylate was in fact found to be more resistant to salt disproportionation than benzocaine mesylate, despite their similar pH_{max} values (37). This work highlighted other contributing factors to salt disproportionation, among them the intrinsic solubility of the free form *relative* to the base (or acid) salt. Indeed for basic compounds, stronger basicity (higher pK_a), higher intrinsic solubility and lower salt solubility all favor salt formation by increasing pH range over which the salt is favored. Accordingly, increasing the intrinsic solubility, decreasing the pK_a and decreasing the salt solubility will favor salt formation for acidic compounds.

While pharmaceutical salts can have lower solubility due to common ion effects, they will as a rule dissolve faster than their conjugate acids (or bases), even at pH_{max} , where the equilibrium solubility is the same (38). In such cases, the salt acts as its own buffer, altering the pH of the diffusion layer surrounding the bulk drug. Supersaturation achieved through self-association of the salt in the region of the pH_{max} may reduce the rate of nucleation of the final solid form (39). Before a highly soluble salt of a poorly soluble compound is selected to enhance bioavailability, the risk of poor physical and chemical stability should be carefully weighed. Not only can highly soluble salts potentially significantly limit the pH range of thermodynamic stability, but they will also increase the thermodynamic driving force for disproportionation over less soluble forms. For these reasons, it is generally advisable to select a salt of *sufficient* solubility as opposed to the one with the greatest solubility. In our experience, excess solubility is likely to cause issues of chemical and physical instability.

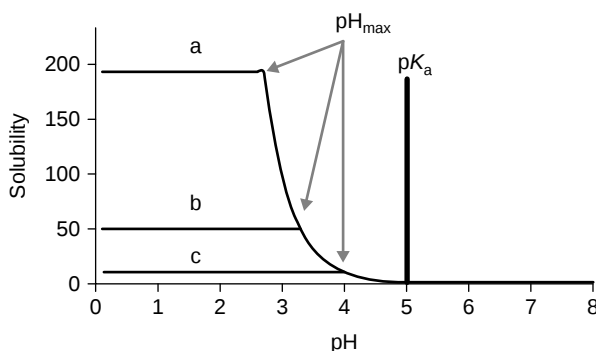


Figure 4 Solubility diagram of salts of a weak base having an intrinsic solubility of 1 mg/mL, and pK_a of 5.0 with salt forms a (hydrochloride), b (sulfate), and c (tosylate), having solubilities of 200, 50, and 10 mg/mL, respectively.

Cocrystals

In finding pharmaceutically acceptable solid-state forms, whether the form is unionized, a salt or cocrystal should not matter as long as its physicochemical properties are suitable for the drug product. Oddly enough, although they have been known for more than a hundred years, only recently has there been a growing acceptance of cocrystals as APIs. The reluctance of the pharmaceutical industry and regulatory bodies to accept cocrystals is somewhat puzzling considering that for acid–base complexes, the lone difference between a salt and a cocrystal is the location of the acidic H atom(s) in the crystal structure. In fact, when the ΔpK_a between an acid and base is less than 3, crystallization may yield salts, cocrystals, or disordered solid forms, with the location of the acidic proton being strongly dependent on the specific crystal packing environment (40). Here, it must be understood that the pK_a value is a solution property that is not specifically defined in crystals. As such, pK_a relationships cannot be transferred to the solid state in a general way (41). Because pK_a values provide an unnecessary limitation to the search for pharmaceutically viable forms, Stahly has proposed omitting the “rule of three” entirely from solid-state screen designs, so that all novel solids, salts and cocrystals alike, are discovered simultaneously, unbiased by pK_a considerations (42).

Cocrystals not only expand the options for altering the solid-state properties of ionizable compounds (by not excluding guests based on pK_a criteria), but they also create opportunities to improve the properties of nonionizable drug substances. Similar to salt formation, cocrystallization has been shown to be effective in improving physicochemical properties, such as hygroscopicity and physical stability (43,44). For compounds that are either oils or amorphous solids, a pharmaceutically acceptable salt or cocrystal would most certainly be welcome to ensure its chemical stability and performance in a solid oral drug product. Such was the case recently for an orally active prodrug of gemcitabine (45). As a *very* weak free base ($pK_a \sim 0.65$), the gemcitabine prodrug was an oil, so 1:1 salts and cocrystals were screened in search of a crystalline form for the isolation, purification and storage of the drug substance. In this case, the preferred crystalline form that came out of the screen was both a salt and a cocrystal formed between *two* equivalents of gemcitabine prodrug per equivalent *p*-toluenesulfonic acid. As shown in Figure 5, protonation of the cytosine ring of the prodrug creates a complementary structure to the neutral molecule, resulting in a second equivalent of gemcitabine prodrug

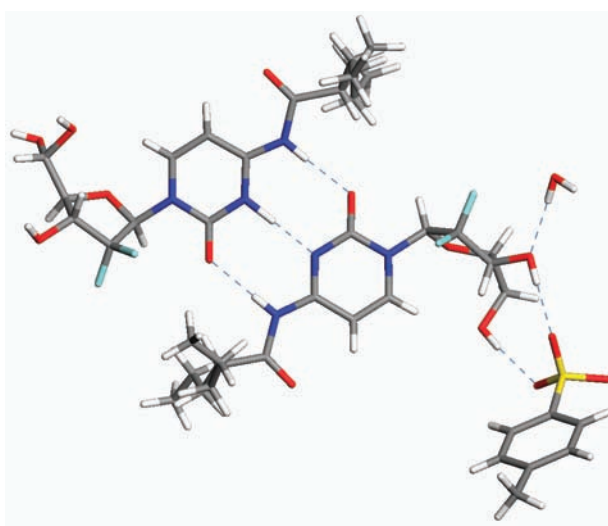
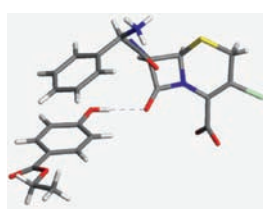


Figure 5 Gemcitabine prodrug hemi-*p*-toluenesulfonic acid hemihydrate salt cocrystal.

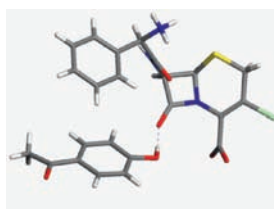
being sequestered in the crystal structure by triple hydrogen bonding, like that observed for the guanine–cytosine (GC) base pair in DNA. Interestingly, hydrogen bonding in the gemcitabine prodrug cytosine–cytosinium ion dimer appears to be stronger (based on shorter intermolecular contacts) than that in the GC base pair.

Much of the growing interest in cocrystallization within the pharmaceutical industry can be traced to the promise that cocrystals hold for enhancing the aqueous solubility of poorly soluble drugs (46). It must be recognized, however, that more soluble cocrystals carry similar risks as more soluble salts for transformation to less soluble (more stable) forms, so understanding the solubility properties of cocrystals relative to their components is paramount to ensuring their performance in a drug product. The aqueous solubility of cocrystals formed between nonionizable components will, of course, be independent of the solution pH. However, when an acidic or basic drug cocrystallizes or a nonionizable drug is paired (cocrystallized) with an ionizable guest, acid–base equilibria will exist in solution, resulting in pH-dependent solubility (47). The phase solubility of cocrystals of ionizable component(s) is analogous to that described for sparingly soluble salts and may be described in terms of the solubility product and ionic equilibria in solution (48). In fact, it was the pH-dependent solubility of the cocrystal formed between the antibiotic, loracarbef, and ethyl paraben that was put to good use in the recovery, isolation and purification of this expensive semisynthetic carbacephalosporin (49). Discovered during the pediatric suspension development of loracarbef (methylparaben was used as a preservative to ensure RT shelf stability), cocrystallization of [carba]cephalosporins and parabens (and hydroxyacetophenone analogs) was found to be surprisingly common, having been observed for several [carba]cephalosporin and paraben analogs. Cocrystallization appears to have been driven not by the strongest hydrogen bonding between the ion pairs, but rather by hydrogen bonding between either the carbonyl O of the β -lactam host and the phenol-OH of the paraben guest or the ammonium NH of the host and the carbonyl O of the guest, Figure 6. Owing to the pH-solubility dependence of the ethyl paraben cocrystal, the β -lactam could be sprung from the paraben cocrystal in high yield through careful adjustment of the pH.

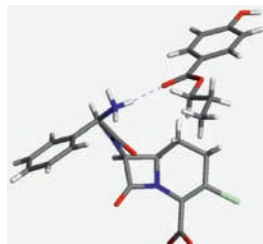
The ability to design molecular solids through cocrystallization with the aim of tailoring specific physical or chemical properties has piqued the interest of many in the growing field



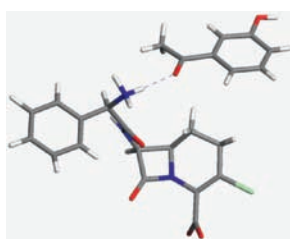
Cefaclor : ethylparaben



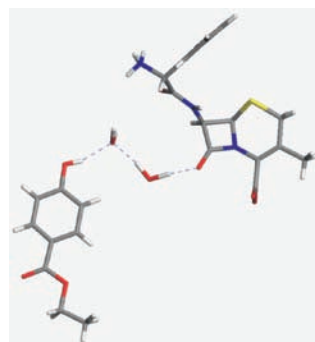
Cefaclor : 4-hydroxyacetophenone



Loracarbef : propylparaben



Loracarbef : 3-hydroxyacetophenone



Cephalexin : ethylparaben : 2 H₂O

Figure 6 Cephalosporin and carbacephalosporin cocrystals.

of crystal engineering in recent years. Indeed, numerous success stories have been reported on “engineering” cocrystals through identifying robust hydrogen bonding synthons of the host, then selecting guests based on statistically-favored hydrogen bonding interactions. Carbamazepine, for example, with its single carboxamide functional group, has been widely studied as a model pharmaceutical for cocrystal formation (50). Compared to most pharmaceuticals, however, carbamazepine is a relatively straightforward candidate for crystal engineering. More typically, drug molecules contain multiple functional groups, which can compete for the same hydrogen bonding sites, rendering hydrogen-bond directed cocrystallization less predictable. Certainly, the cocrystallization of the gemcitabine prodrug *p*-toluenesulfonic acid salt with a second equivalent of the prodrug had admittedly not been by design. Had parabens been so evident a cocrystal former with loracarbef, it is unlikely they would have been used as preservatives in the pediatric suspension formulation of this antibiotic either. In the end, just as pK_a criteria may unnecessarily limit the design space explored in search of pharmaceutically-useful crystalline forms, so also may placing too much weight on hydrogen-bonding interactions to single functional group synthons to drive guest selection for cocrystallization. For this reason, whether the goal is to identify novel crystalline forms of nonionizable molecules or to expand the scope of a crystallization screen beyond salts (by omitting the rule of three), cocrystal screen designs should include acidic or basic guest molecules not otherwise used in “salt” screening, as well as common additives and excipients used in the food and drug industries.

Polymorphs and Solvates

Crystal polymorphism and hydrate formation are central to the selection of the preferred crystalline form, be it of the parent molecule, a salt or cocrystal, of the active pharmaceutical ingredient. Polymorphism, a long time interest, if not curiosity, to crystal chemists, has drawn considerable attention in recent years by the pharmaceutical industry, as well as regulatory bodies. The FDA, recognizing that different crystalline forms of the same compound may differ in physical properties that have the potential to affect the quality or performance of drug products, published guidance in 2000 for setting and justifying acceptance criteria for new drug substances and drug products produced from them. The International Conference on Harmonisation (ICH) Guidance on Q6A Specifications states that when physical property differences between forms are shown to affect drug product performance, bioavailability or stability, the appropriate solid state should be specified (51). More recently, the FDA in publishing ICH Q8 guidance stated that quality should be built into drug products by design as opposed to being tested into them (52). Not surprisingly, the quality by design (QbD) approach to pharmaceutical development has identified solid form as a potential critical quality attribute to ensuring product quality and performance. Clearly, today’s regulatory climate underscores the importance of carefully and thoroughly screening for and evaluating the physicochemical properties of drug crystal forms.

As mentioned previously, the diversity of solid forms observed for most drug molecules can be traced to both conformational flexibility (conformational polymorphism) and a combination of intermolecular forces, including ionic, van der Waals, dipole–dipole and hydrogen-bonding interactions, which in crystallization allow molecules to assemble in different ways to minimize the system’s free energy. For molecular solids dominated by isotropic ionic and van der Waals interactions, the most stable structure energetically should be that which is most efficiently packed, that is, most dense (close packing principle). When other interactions, for example, hydrogen bonds, dominate the packing, large voids can be created in the crystal to satisfy the orientational requirements imposed by certain motifs, leading to energetically-favorable, lower density structures.

Polymorph (and solvate) screens should reveal the complexity of the solid form landscape of the drug candidate, enabling crystal forms to be identified that are sufficiently stable and capable of consistently delivering the drug to the patient. But how many forms are likely

to exist and what assurance is there that the most stable of the forms has been found? Computational studies of crystal energy landscapes have shed light on the subject by showing that many thermodynamically competitive crystal structures resulting from both conformational flexibility and multiple hydrogen-bonding topologies may exist within approximately 8 to 10 kJ/mol of the most stable form (53). For example, the computed crystal energy landscape of the diuretic drug hydrochlorothiazide (HCT) is comprised of nearly 60 energetically feasible crystal structures, in this case within 12 kJ/mol of the lowest energy structure, Figure 7 (54). Clearly there are not 60 neat (nonsolvated) polymorphs of HCT. In fact, only two are known, both of which were happily among the packing arrangements identified from the crystal structure prediction. The results of the HCT structure prediction are typical in the sense that the number of energetically feasible crystal structures identified as minima on the crystal energy landscape greatly exceeds the number of observed forms. Although the question will always remain as to how thoroughly crystallization “space” was experimentally explored in searching for polymorphs, structure predictions will inevitably yield many more theoretical structures than the number of experimentally accessible forms. This is because the structure prediction process itself yields energy minima without regard to energy barriers to conversion to more stable forms. In other words, among the polymorphs identified in crystal structure searches, only those with sufficiently high energy barriers to transformation to more stable forms are likely to be experimentally accessible. As for identifying the most stable form, the structure search conducted for HCT was unable to reliably rank the energies of the two known forms, with the metastable Form II calculated to be more stable than the stable Form I. While most crystal structure prediction methods approximate lattice energy or enthalpy (free energy at 0 K), it is the free energy landscape at temperatures amenable to crystallization that is of particular interest and remains a challenge in computational chemistry today.

As the ability of computational methods to correctly rank the relative energies of different packing arrangements at temperatures relevant to crystallization improves and multicomponent systems (including hydrates) become more computationally accessible, the future of crystal

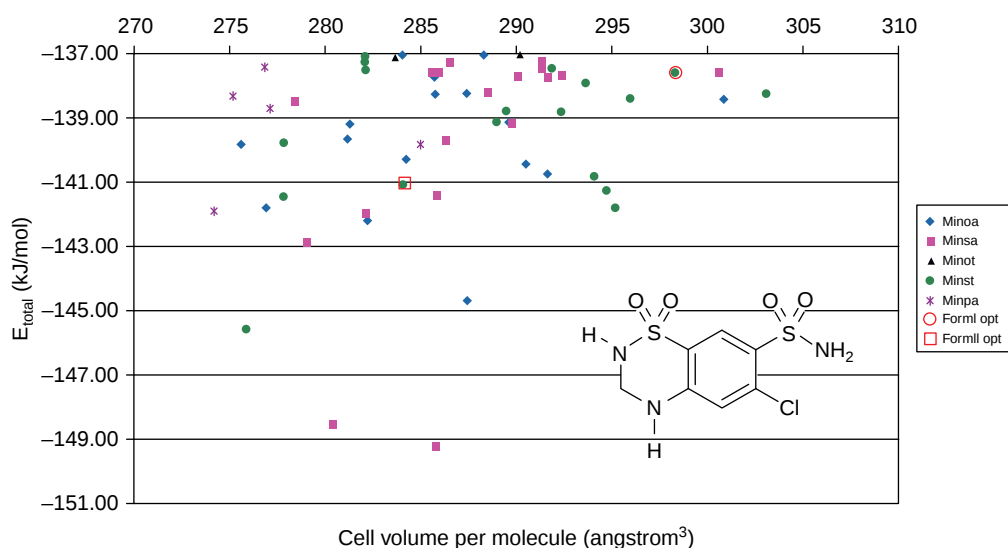


Figure 7 Crystal structure energy (E_{total}) of predicted (closed symbols) and observed (open symbols) hydrochlorothiazide crystal polymorph structures as a function of the unit cell volume per molecule. The calculated structures are color-coded for the rigid molecular conformations used in the structure prediction. Source: Adapted from Ref. 54. Copyright © American Chemical Society.

structure prediction is very bright as a complementary method to experimental solid form screening for identifying viable crystalline forms (55–57). If, for example, more stable structures appear to be possible, continued experimental searching for stable structures may be warranted. Conversely, in rare cases where but a few energetically feasible forms have been predicted, experimental solid form screening may be halted once those forms have been found. Today, however, with no obvious endpoint to solid form screening, that is, no guarantee that other [more stable] forms will not appear at a later time, how extensive should screens be to minimize the risk of late appearing forms? McCrone provocatively stated that “every compound has different polymorphic forms and that, in general, the number of forms known for a given compound is proportional to the time and money spent in research on that compound” (58). This statement can rightfully be extended to solvates, hydrates included. Throughout the years, a number of attempts have been made to determine the prevalence of both polymorphism and solvate formation in organic compounds in general and for drug compounds specifically. Of all compounds, organic and otherwise, the number of polymorphic structures in the Cambridge Structural Database (CSD) as of January 1, 2009 (469,611 entries) was 15,316, representing only 3.3% of the total entries (59). Polymorphism and hydrate formation was found to be much more prevalent among the drug compounds listed in the Pharmacopoeia European (PhEur), with 58% of the 808 compounds having been identified in polymorphic and hydrated crystal forms (60). Surveys of the CSD, which include all organic compounds, as well as those of drug substances accumulated over decades, are likely to underestimate the prevalence of crystal polymorphism and hydrate formation under experimentally accessible conditions. Stahly, in tabulating the results of 245 variably scoped “polymorph” screens conducted at SSCI, Inc., recently reported that about 90% of compounds screened were identified in multiple solid forms, crystalline and amorphous (42). True polymorphic forms were found in about half of the screens, while about a third of the compounds formed solvates, including hydrates. The greater tendency of salts to crystallize as solvates (hydrates) and nonsalts to exhibit polymorphism was noted in both the PhEur and SSCI surveys.

Early in drug development, solid-state research may be focussed on identifying even one sufficiently stable solid form, and in some cases one that is enabling, to ensure adequate exposure with minimum variability. In recent years, slurry (“stable form”) screening has become immensely popular for finding stable polymorphs and hydrates. The slurry technique entails suspending a less stable amorphous or crystalline form in a saturated solution of the drug and allowing the more stable form to crystallize at the expense of dissolving the less stable form. Since the rate at which a solution-mediated phase transformation occurs will depend on both the dissolution kinetics of the metastable form and the nucleation and growth kinetics of the stable phase, the solubility of the drug substance in the medium is an important factor in driving the transformation in a reasonable timeframe. Having observed generally faster nucleation rates for polymorph conversions in solvents giving higher solubility, Gu and Grant have shown that a minimum solubility of about 1 mg/mL in a solvent should reduce the interfacial tension between solute and solvent sufficiently to successfully mediate phase transformations (61). The slurry conversion technique, while generally effective in finding stable forms, will not always produce the most stable form, however. As shown in Figure 8, if Form B is crystallized first and the critical free energy barrier of nucleation is not overcome for transformation to the more stable Form C, Form C will not be observed. Thus, while screening for stable forms by slurry methods may suffice to progress compounds in early clinical development, the search for *all* pharmaceutically relevant crystal forms, those forms with the potential to be intentionally or unintentionally introduced in the drug product, will ultimately encompass many methods, including solution crystallization, thermal and RH annealing, and mechanical stressing (62).

Crystallization from solution by solvent evaporation, cooling and antisolvent addition is extensively used in comprehensive screens for polymorphs and solvates. Here, diverse solvent property space is usually explored based on the premise that the success rate of discovering new solid forms may be increased if solvents with diverse properties are surveyed (63). To this

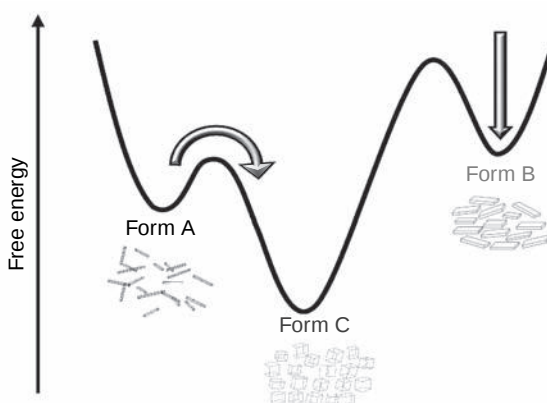


Figure 8 Schematic crystal energy landscape showing barriers to transformation to the thermodynamically stable form, Form C. If the first form that crystallizes from solution is the kinetically favored metastable polymorph, Form B, the thermodynamically stable Form C may be inaccessible via solution-mediated phase transformation.

end, high throughput methods have been employed using integrated robotics for material generation on small (μg -mg) scale, x-ray powder microdiffraction and/or Raman microscopy for characterization, and software tools for sorting, analyzing and reporting data. Automated methods allow well-behaved drug substances, which readily crystallize, to be rapidly screened. Relying solely on high-throughput automation is, in the authors' experience, neither efficient nor altogether effective for comprehensive form screening, however. Aside from the difficulties frequently encountered in reproducing and up-scaling μg experiments, the solubility properties of the drug substance are rarely accounted for in mass parallel crystallization designs and drug crystal forms are notorious for failing to nucleate from solution in the timeframe of programmed experimental routines. In cases where the solubility properties are not accounted for in crystallization and proper measures are not taken to completely dissolve the drug substance (to eliminate seed crystals of the starting crystal form), crystallization screens will at best yield the starting form or one of the known stable forms likely already identified from initial slurry screening. Slow crystal nucleation and growth, on the other hand, may result in oiling, emulsions and precipitation of amorphous phases from the highly supersaturated solutions that are inevitably generated. Finally, it may be argued that thousands of experiments are not needed to find polymorphs and solvates. In most cases, a series of well-designed (tailored to the solubility characteristics of the API) and carefully executed crystallization experiments will produce more useful results, that is, more forms, than mass parallel crystallization screening from but a few operating conditions.

For some compounds, crystalline forms suitable for a commercial drug product will be obtained from either the very first attempts to isolate the drug substance or they will readily fall out of automated screens. In other cases, the process of finding a viable crystal form is arduous and lengthy. The recent discovery of a conventional mono-HCl salt of N-[(1*S*)-1-[2-(1-[(3*S*,4*R*)-1-*tert*-butyl-4-(2,4-difluorophenyl)pyrrolidin-3-yl]carbonyl)piperidin-4-yl]-5-chlorophenyl]ethyl]acetamide, which occurred after almost two years of searching for pharmaceutically-viable forms, attests to the sometimes long and serendipitous path to finding suitable solid-state forms (64). Clearly there is no guarantee that crystalline forms of the parent compounds, their salts or cocrystals will ever be found, let alone be useful in a drug product. In some instances, perfectly viable forms may only be found with either careful control of crystallization conditions or changes in the purity (or impurity profile) of the starting material. What must be recognized in solid-state form screening of any kind is that because all compounds are unique, there is not a single surefire approach to finding drug crystal forms. Once again, the

quality of a solid-state screen, particularly for polymorphs and solvates, is more easily gauged by the number and diversity of well designed and executed crystallization attempts than by the number of forms found. This underscores the importance of documenting all attempts made in search of solid-state forms.

Amorphous Forms

Amorphous forms are the most energetic of solid-state forms, possessing greater specific surface area and lacking the lattice energy of their crystalline counterparts, which makes them attractive for improving the bioavailability of compounds with dissolution rate limited absorption. More than 40% of oral drug products contain poorly soluble drugs, and among the pharmacopoeia, this share is more than 30% (65,66). For BCS class II drugs with low solubility and reasonable permeability, dissolution is the rate-limiting process of drug absorption and enabling technologies, which rely on rendering the drug candidate amorphous and capitalizing on the enhanced solubility, may be the only choice for successful development of a drug candidate. The true solubility enhancement provided by amorphous phases has been difficult to assess, however, owing in part to spontaneous crystallization during measurement (26). The development of amorphous forms also presents significant challenges in reproducibly manufacturing the amorphous state and carries a risk of "catastrophic" crystallization downstream. Whether the amorphous phase is intentionally present for the purpose of attaining greater bioavailability or unintentionally present as a "nuisance" phase, a basic understanding of the chemical reactivity and phase behavior of amorphous forms is essential to understanding and controlling the physical properties of any pharmaceutical.

Noncrystalline character arises in many ways and even well-ordered single crystals are imperfect as misalignment of one unit cell to the next will create a certain mosaicity within a single crystal. Crystallographic defects occur where one plane of the lattice is slightly out of alignment with respect to the next, while line defects arise from a slip process induced by shear stress resulting in lines of unit cells being out of position with respect to the remainder of the crystal. Line defects take on two basic forms, edge dislocations or alternatively screw dislocations. Point defects are vacancies within the crystal caused by impurities incorporated into the lattice. These dislocations result in strain energy relative to the ideal crystal (67). Within the lattice itself, there can be disorder associated with atoms or groups of atoms not taking a perfectly reproducible position from one unit cell to the next, a type of disorder commonly associated with solvent molecules within a lattice or aliphatic chains, disorder which is static in nature (68,69). Other disorders are dynamic. Common motions that occur within the lattice involve motion of individual atoms or groups of atoms, even aromatic ring flipping can commonly be accommodated within the space of the crystal lattice. Depending on the frequency of the motion, such motions can be studied by solid-state NMR spectroscopy. Such disorders may also give rise to a reduction in the intensity of coherent scattering of x-rays by a crystal, but should be distinguished from the amorphous state. While it is not uncommon for these types of disorder to be characterized as amorphous, they are different and might result in quite different physical properties or behaviors (70). The following discussion focuses primarily on amorphous materials which lack three-dimensional periodicity (long-range order), do not diffract x-rays or melt, and exhibit a characteristic glass transition.

A complicating factor in dealing with amorphous or partially amorphous materials is that the amorphous state is not truly an equilibrium physical state, but instead represents a continuum of disordered states, where molecules are kinetically trapped. As such, the thermodynamic variables (entropy, enthalpy, volume) of a glass will depend on its thermal history. When a liquid is cooled below the melting point, T_m , either crystallization can take place via a first order transition, thereby reducing its specific volume (with water being one noted exception) and enthalpy, or the liquid can become supercooled. In the latter case, the specific volume (and enthalpy) of a supercooled liquid will continue to decrease as the temperature is decreased below T_m , Figure 9. At the temperature where the viscosity of the supercooled liquid becomes

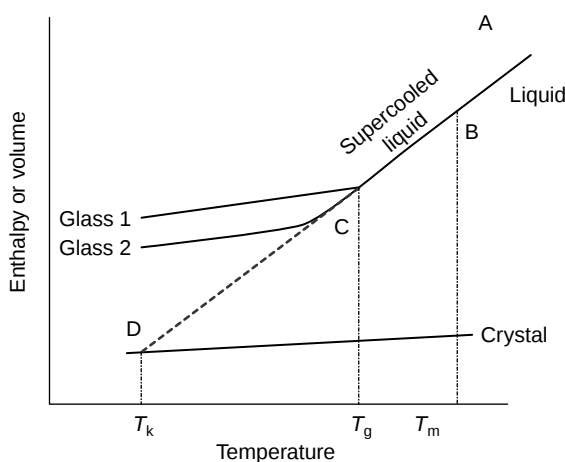


Figure 9 Illustration of the change in enthalpy or volume as a supercooled liquid is cooled below T_g .

so great that large-scale molecular rearrangements are either no longer possible or not sufficiently fast to maintain an ideal supercooled state, a second order transition occurs and the structure becomes “frozen in.” Below this temperature, known as the glass transition temperature, T_g , the specific volume or enthalpy will deviate from that of the ideal supercooled liquid and the viscosity will dramatically increase (71).

The stability (or lack thereof) of amorphous forms will strongly depend on relaxation processes, which occur in the supercooled liquids. With aging, a glass that is initially formed, glass 1, may relax to a lower energy glassy state, glass 2, at a rate characterized by relaxation time τ (72). The most studied relaxation process, α relaxation, involves large angular or orientational movements and/or translational motions over sizeable distances. This type of structural relaxation is characteristically non-Arrhenius and is predominantly observed at temperatures above T_g , where diffusion rates are higher. The less studied β relaxation becomes more apparent below T_g . This type of structural relaxation is characteristically Arrhenius and involves smaller angular reorientation (e.g., methyl group rotation, aromatic ring flipping) on a much shorter timescale or higher frequency than α relaxation (73). What is particularly noteworthy about β relaxation involving whole-molecule reorientation (Johari–Goldstein-type motion) is its apparent correlation with nucleation (clustering of molecules) (74,75). Although crystallization is generally viewed as a two stage process involving nucleation and growth, it has been theorized that crystallization from glasses with undercooling is nucleation controlled. In general, both nucleation and growth rates will be faster above T_g , however nucleation rates peak at lower temperatures than crystal growth rates due to the reduced dependence on bulk diffusion to the growing crystal surface. This is illustrated schematically in Figure 10. The thermodynamic driving force for crystallization, given by the Gibbs free energy difference between the melt and crystal, ΔG_v , may be approximated (assuming that the difference in heat capacity is constant) using the Hoffmann equation, emphasizing the importance of the degree of supercooling ($T_m - T$) as shown in Eq. (2).

$$\Delta G_v = \Delta H_f (T_m - T) T / T_m^2 m \quad (2)$$

Reliably predicting the physical stability of an amorphous form, which is difficult due to the stochastic nature of the crystal nucleation process, is confounded by the fact that different amorphous materials are formed, depending on the method of preparation and the thermal history, and enthalpic relaxation may occur over time, affecting molecular mobility and the tendency of a glassy solid to crystallize (77–80). It is a commonly held belief that amorphous

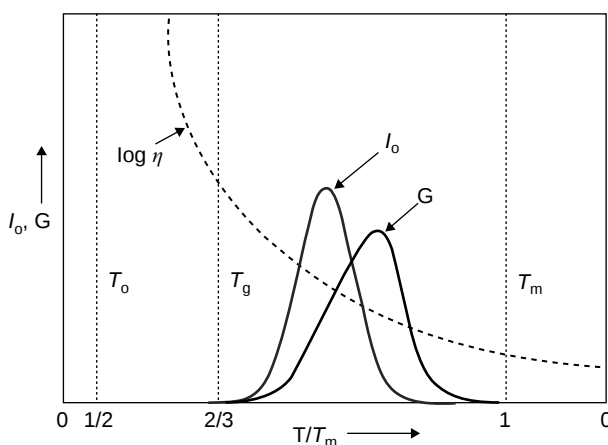


Figure 10 Schematic of crystallization from a supercooled liquid, showing the temperature dependence of the nucleation rate I_o , crystal growth rate G , and viscosity η . T_o is the VTF zero mobility. Image recreated based on original work of Gutzow (76).

solids are less susceptible to crystallization when stored below the T_g . While it is true that a substance kept dry and below its T_g is less likely to crystallize, crystallization has been observed at temperatures significantly lower than T_g . Hancock et al. in a study of the molecular mobility of three different glassy compounds, concluded that storing compounds 50°C or more below their T_g was sufficient to render molecular motions responsible for compromising physical stability negligible over the lifetime of a typical pharmaceutical product (81).

Understanding the phase stability of an amorphous form alone is insufficient to ensure its physical stability in a drug product, where it will inevitably be mixed with other substances which can either promote or inhibit crystallization. It has long been known, for example, that water acts as a plasticizing agent, which when absorbed into an amorphous solid, lowers its glass transition temperature and increases molecular mobility leading to enhanced rates of crystallization (82). Imaizumi et al. first reported that amorphous indomethacin held at 30°C and exposed to high RH exhibited a greatly enhanced rate of crystallization (83). Zografis and coworkers later found that the T_g decreased approximately 10°C for every 1% increase in water content (84). The T_g lowering effect of plasticizers, such as water, may in fact be predicted assuming ideal mixing by the Gordon–Taylor equation [Eq. (3)],

$$T_{g \text{ mix}} = (x_1 T_{g1} + K x_2 T_{g2}) / (x_1 + K x_2) \quad (3)$$

where $T_{g \text{ mix}}$ is the glass transition of the molecular mixture, T_{g1} and T_{g2} are the glass transition temperatures of the individual components, and x_1 and x_2 are their molar fractions, respectively. As K is related to the ratio of the free volumes of the two components, the plasticizing effect of diluents is expected to decrease with increasing molecular weight (85,86). For drug substances that are particularly prone to crystallization, it follows that polymers may be added to inhibit crystallization by increasing T_g . A study of PVP mixtures with indomethacin illustrates the predictable influence of miscible, higher molecular weight polymers and its stabilizing (antiplasticizing) effect on mobility. Experimental T_g values for blends of indomethacin and different molecular weight PVP were compared to those calculated using the Gordon–Taylor equation (87). While the agreement between the experimental and calculated T_g values was reasonably good, greater stability was afforded by high molecular weight PVP than predicted by the Gordon–Taylor equation, a stability enhancement attributed to hydrogen bonding interactions between the polymer and drug. While the Gordon–Taylor equation accounts for many systems reasonably well, the assumption of ideal mixing is rarely realized in practice,

so modifications have been made to the basic equation in an attempt to account for specific interactions causing deviations from ideality (88).

The use of polymers to stabilize amorphous pharmaceuticals has increased dramatically in recent years in an effort to enable greater exposure through formation of solid dispersions (89). Sekiguchi et al., first recognized the potential for bioavailability enhancement using polymers in their study of eutectic mixtures of sulfathiazole (90). Later, when the inherent advantages and liabilities of the solid dispersions were explored, a 700-fold enhancement of dissolution rate was noted, which decreased over time. Using thermal analysis and x-ray powder diffraction, the slower dissolution rate was traced to crystallization of sulfathiazole from the solid dispersion, highlighting the potential risks of this bioavailability enhancement approach (91). Clearly, having the ability to judge the feasibility of an amorphous form, that is whether or not it will have sufficient physical stability to be maintained throughout the shelf life of the product, is essential. In the end, to minimize the risk of crystallization, an amorphous form, alone or as a solid dispersion, should be selected having a T_g at least 50°C above the intended storage temperature.

Amorphous forms are likely to be suitable for drug products only in cases where both chemical stability and physical stability with respect to crystallization can be demonstrated. Initial solution-state stress testing can provide useful insight into the feasibility of amorphous forms. If, for example, a drug substance is found to be reactive in solution, then it is unlikely that an amorphous form will be sufficiently stable; a crystalline form will most always be the preferred solid-state option. Accelerated stability studies may be conducted to predict the chemical and physical stability of amorphous forms; however, it is important to recognize that behaviors above and below the T_g of an amorphous form may not be comparable (92). Therefore, it is imperative that the T_g be assessed in relation to the storage temperature. The influence of moisture on the T_g should also be evaluated (93). An important consideration in any form of amorphous stress testing is whether the amorphous material remains amorphous throughout the course of the study. Techniques used for the solid-state characterization of structure (or lack thereof), mobility, thermodynamic properties, and relaxation in amorphous materials are summarized in Table 1. Strategies used for characterizing amorphous solids, which are necessarily different from those used for crystalline phases, have been thoroughly reviewed elsewhere (94–96).

Table 1 Physical Techniques for Characterizing Amorphous Solids (80)

Technique	Information
X-ray diffraction (XRD)	DOC, CK
Molecular spectroscopy	SR (e.g., Raman and NMR), microheterogeneity
Differential scanning calorimetry (DSC)	DOC, microcrystalline or truly amorphous, CK, SR
Isothermal calorimetry	SR, CK, DOC
Modulated DSC (MDSC)	Reversing vs. nonreversing heat flow, c_p , SR (0.1–0.01 Hz)
Solution calorimetry	Excess enthalpy, DOC
Adiabatic calorimetry	Excess enthalpy, entropy and free energy
Dielectric analysis (DEA)	SR, primary vs. secondary processes
Dynamic Mechanical Analysis (DMA)	SR
Viscometry	SR
Dilatometry	T_g , liquid/glass expansion coefficient
Solubility	Excess free energy
Density	Density difference from crystalline solids
Thermally stimulated current (TSC)	SR, DOC, microheterogeneity
Water vapor sorption (gravimetric)	Hygroscopicity, DOC, CK

Abbreviations: CK, Crystallization kinetics; DOC, Degree of crystallinity; SR, Structural relaxation (T_g , τ versus T , fragility, etc.)

ENSURING CHEMICAL STABILITY AND PHYSICAL INTEGRITY OF PHARMACEUTICAL SOLIDS IN DRUG PRODUCTS

Limited solid form screening is generally conducted early in drug development not only to identify, perhaps for the first time, crystalline forms of drug candidates, but also to improve the solubility characteristics of poorly water soluble compounds. Crystalline form selection at this stage is frequently driven by the solubility needed to achieve sufficient exposure for a margin of safety to be established and to gain a better understanding of the doses necessary to achieve efficacy. Due to the timing of the salt screening activities and insufficient and oftentimes non-representative material funding this research, salt selections in the lead optimization stage of development are not always based on “representative” samples of the salt hits and rarely are they made with full knowledge of the solid form landscape. Indeed, new forms appear all too frequently as process chemistry is optimized, key impurities are removed and other new impurities appear. Once the liabilities of the initial salt crystal form are revealed with further research, alternative forms may be pursued for pivotal clinical trials and ultimately the commercial drug product (97). Changing solid forms is a costly, but in some cases, necessary undertaking, which will require redeveloping new crystallization and formulation processes, repeating toxicology and stability studies, establishing bioequivalence, potentially adjusting dose strengths, any of which may add to development timelines. Progressing a suboptimal solid form into commercial drug product development, on the other hand, can be an expensive proposition itself and carries the ultimate risk of product failure once on the market (98).

To select the optimal crystal form for an orally administered drug product, both the manufacturability and physicochemical properties, including physical and chemical stability under processing and storage conditions, of the viable forms must be established. Controlling which solid-state form will emerge from a crystallization process and the extent to which impurities are rejected in the crystallization of a drug substance requires selecting between competing crystallization pathways that are governed by thermodynamics, crystal nucleation and growth kinetics and molecular recognition events (99–101). Key aspects of drug development to ensure the chemical stability and physical integrity of drug substances and products from a solid-state perspective are described herein, including:

- evaluating the stability of forms comprising the solid form landscape of the drug substance to ensure robust form selections and to understand the risks of physical changes under storage or stress conditions,
- assessing chemical stability and physical integrity of the drug crystal form alone and in the presence of excipients during formulation processing and product storage.

Selecting Drug Crystal Forms

When multiple pharmaceutically relevant (anhydrous or hydrated) crystal forms of a drug compound, its salts and/or cocrystals are identified, a multidimensional evaluation of physical properties (solubility, stability, hygroscopicity, etc...) and manufacturability should be performed to select the preferred solid form (102). In principle, a solid form need only be kinetically stable throughout formulation processing and the shelf-life of a drug product, however, the thermodynamically stable form, that with the lowest free energy, is generally preferred to mitigate the risk of undesired process- or storage-induced phase transformations. If the solubility properties (and dissolution rate) of a given free acid, base or salt in its thermodynamically stable crystal form are not sufficient to deliver the drug substance at an efficacious dose, particle size reduction, different solid forms and formulation options must be considered. As dissolution rates depend on both solubility and surface area, milling or micronization can effectively increase the surface area to enhance delivery of the drug. Milling or micronization carries risks of amorphization, however, there is a practical limit to how small particle size can be reduced and maintained. To increase the dissolution rate (saturation solubility) of a drug, the diversity of solid-state forms (Fig. 1), including enabling metastable crystal forms, alternate salts or cocrystals, and even amorphous forms, may be exploited. Amorphous and

metastable crystalline forms provide a solubility (and dissolution rate) advantage over thermodynamically stable forms, however, only by exception, will the more soluble forms be sufficiently resistant to converting to less soluble crystalline forms. Alternate salts and cocrystals are attractive options for increasing saturation solubility, but the general preference for the thermodynamically stable form remains. Thus, except in cases where a robust crystallization process or enhanced solubility/dissolution behavior required for bioavailability cannot otherwise be achieved, the thermodynamically most stable form under processing and storage conditions is selected for orally administered drugs.

Given the strong preference to select the thermodynamically stable crystal form, form selection oftentimes amounts to determining which free/salt/cocrystal form of a drug substance in its most stable (anhydrous or hydrated) crystal form best meets the requirements for the drug product in terms of physical and chemical stability, solubility, dissolution rate, hygroscopicity, morphology, physicomachanical properties, etc. Crystal polymorphism and solvate formation can factor into the selection of a free form, salt or cocrystal for the commercial drug product. Polymorphism can certainly frustrate attempts to directly crystallize the thermodynamically stable form and is especially problematic in the case of enantiotropic polymorphs (*vide infra*) when the thermodynamic transition temperature lies near the storage temperature. Hydrate formation can be cause for concern because different hydrated forms may be thermodynamically stable depending on the RH and changes in hydration state can significantly alter the solubility properties of the drug substance. In these cases, the final choice between anhydrides and hydrates must consider the thermodynamic, as well as the kinetic, stability of the respective forms at the RHs encountered during normal storage and handling of the drug substance and formulated product (103). Organic solvates, rarely used in drug products, but frequently encountered in crystallization screening and process development, can be problematic when they severely limit the crystallization conditions under which the preferred form can be obtained.

Selecting the most stable crystal form of a free acid/base, salt or cocrystal will mitigate any risk of conversion to more stable forms (assuming of course that the most stable form has been identified); however, it will not guarantee that over the shelf life of the drug product, conversion to the absolute thermodynamic minimum, such as a salt of different stoichiometry or free acid/base form, will not occur. For example, in cases where the salt form is thermodynamically less stable than its conjugate free acid or base in the pH microenvironment of a solid oral dosage form, then the salt will be at risk for disproportionation. Here, the greater the difference in solubility of the salt versus its free form, the greater the free energy difference driving disproportionation. Clearly, not only should the risks of polymorph transformations and hydration events be considered in the selection of the solid-state form, but so too should potential conversions to different salts or free forms when in the formulated drug product. As will be discussed later, excipients may be used to control the pH microenvironment to minimize the risk of disproportionation.

To identify the thermodynamically stable crystal form of a drug substance under typical storage conditions and to assess the risks of physical changes during processing, a phase diagram must be at least semiquantitatively understood as a function of temperature and where hydrates are known to exist, as a function of RH. A careful assessment of form stability is therefore a necessary first step following the discovery of solid-state forms to ensure robust form selections, as well as being a prerequisite to designing crystallization processes to select for the preferred form and identifying storage and processing conditions to preserve the selected form. Evaluating the stability of crystal forms comprising the oftentimes complex solid form landscapes of drugs is not without its challenges, however. Not only are the free energy differences between drug crystal forms frequently small, which makes reliably assessing stability difficult, particularly when measurements are made well outside the temperatures of interest, but drug substances and their crystal forms are also notorious for transforming to other crystal forms under the conditions used to evaluate form stability. Just as there is no single surefire approach to finding forms, no single technique is universally effective in evaluating form stability for all drug substances. Fortunately, several methodologies are available to either directly or indirectly measure the solid-state stability of drug crystal forms, qualitatively or quantitatively, Table 2 (104).

Table 2 Direct and Indirect Methods to Evaluate Solid-State Stability of Drug Crystal Forms

Method	Measurement	Information	Temperature Range	Advantages	Potential Problems
Slurry bridging	Suspension phase composition	Rank order of G	T_m to T_{bp} of solvent, up to T_m of drug	Simplicity	Solution-mediated phase transformations to other forms, labile solvates, slow transformation kinetics due to poor solubility or when near T_i
Equilibrium solubility	Solution concentration, suspension phase composition	ΔG ; T_i by extrapolation	10 to 60°C	Direct measurement of quantitative ΔG	Solution-mediated phase transformations, non ambient temperature control throughout measurement
Kinetic solubility	Dissolution temperature with heating	ΔG ; T_i by extrapolation	10 to 100°C	Rapid analysis, automated measurement of solution clear points	Thermal lag with fast heating, solution-mediated phase transformations with slow heating
Intrinsic dissolution rate	Dissolution rate from constant surface area	ΔG ; T_i by extrapolation	10 to 60°C	Yields quantitative ΔG before form conversions occur in suspensions	Compression-induced phase transformations
DSC	Melting point heat of fusion	ΔH (monotropy v. enantiotropy via heat of fusion rule); T_i by extrapolation	Near T_m	Small sample size, rapid analysis	Phase transformations below T_m , melting with decomposition, requires highly crystalline, phase pure samples
	Heat of transition	ΔH (monotropy v. enantiotropy via heat of transition rule)	Near T_i	Small sample size, rapid analysis	Reproducibility, not accessible for all polymorph pairs—only relates forms involved in transition
Solution calorimetry	Heat of solution	ΔH (monotropy v. enantiotropy via heat of solution rule)	0 to 40°C	Yields ΔH for compounds which melt with decomposition	Larger sample size, solution-mediated phase transformations
Moisture sorption analysis	Moisture sorption isotherm	Rank order of G v. RH or α_w , critical water activity (α_{crit})	RT to 60°C (automated analysis); extended temperature range with RH chambers	Direct measurement of α_{crit} in the absence of hysteresis, precise control of RH, measurement can be automated	Only sensitive to phase changes with accompanying weight gain/loss, hysteresis, slow transformation kinetics

The most clear and direct way to quantitatively assess thermodynamic stability is to measure equilibrium solubility, since the Gibbs free energy difference (ΔG) between two forms, I and II, at any given temperature is directly related to the ratio of their solubilities, S_I and S_{II} , as given by Eq. (4):

$$\Delta G_{II-I} = -RT \ln (S_{II}/S_I) \quad (4)$$

Equilibrium solubility measurements are not always feasible; however, owing to solution-mediated phase transformations that occur before the equilibrium solubility of the metastable form is attained. As a surrogate for equilibrium solubility, the intrinsic dissolution rate (IDR) of the crystal forms may be measured. Intrinsic IDR measurements entail compacting the crystal forms into disks to normalize their surface area, then measuring the rate of dissolution under sink conditions. As with the equilibrium solubility approach, the free energy difference between two crystal forms at a given temperature may be calculated from the ratio of their IDRs. The crystal forms must, of course, survive the initial compaction process in order for intrinsic dissolution measurements to give meaningful results. When solution-mediated phase transformations prevent the equilibrium solubility of metastable crystal forms from being measured or drug crystal forms are compromised upon compaction so that IDR measurements become prohibitive, the kinetic solubility of the forms of interest can be measured. With this approach, suspensions of the crystal forms are not intentionally allowed time to reach a thermodynamic equilibrium. Instead, they are heated at a constant rate to achieve dissolution (usually observed by in situ measurement of turbidity). Provided that the heating rate is sufficiently slow, the approximate solubility of each crystal form can be determined at the temperature where the clear point of the solution is observed. This generally automated method can rapidly yield semiquantitative solubility values across a range of temperatures and is oftentimes sufficient to assess relative thermodynamic stability.

Thermal analysis can provide both qualitative and quantitative information about the relative stability of neat polymorphic modifications, the energies associated with their phase transformations, and the monotropic (irreversible) and enantiotropic (reversible) nature of those transitions. For some polymorph pairs, monotropy and enantiotropy can be readily discerned by the nature (endothermic or exothermic) of the transition between the forms. According to Burger's heat of transition rule, the transition from the lower melting to the higher melting form upon heating is endothermic above the thermodynamic transition temperature of enantiotropic forms and exothermic for monotropic forms (105). Since a higher melting enantiotropic form may undergo an exothermic solid-state transition to the low melting form below the thermodynamic transition temperature (the high melting form is less stable than the low melting form below T_I), the appearance of an exothermic solid-state transition is not in and of itself sufficient to establish monotropy. The order of melting must first be established.

When polymorphs fail to readily transform or the transition enthalpy is too small, melting temperatures and enthalpies can be used to indicate monotropy and enantiotropy. In accordance with Burger's heat of fusion rule, the stability relationship is one of monotropy when the higher melting form has the higher heat of fusion and enantiotropy when the heat of fusion is lower. Application of the heat of fusion rule not only requires that each polymorph be isolated in highly crystalline and phase pure form, but also that both the melting points and the enthalpies of fusion are experimentally accessible. When the heat of fusion cannot be measured because of decomposition with melting, for example, the enthalpy difference between the forms can be measured well below the decomposition temperature by solution calorimetry. Monotropy and enantiotropy are inferred in these cases from the relative heats of solution and melting temperatures by applying the analogous heat of solution rule. The calorimetrically accessible thermodynamic quantities that are relevant to deducing the free energy relationships between polymorphs are depicted in Figure 11 (106). While the Burger rules have been widely used to qualitatively interpret DSC data in terms of polymorph stability, Yu has shown that pure and eutectic melting data obtained by DSC can also be used to quantitate the free energy

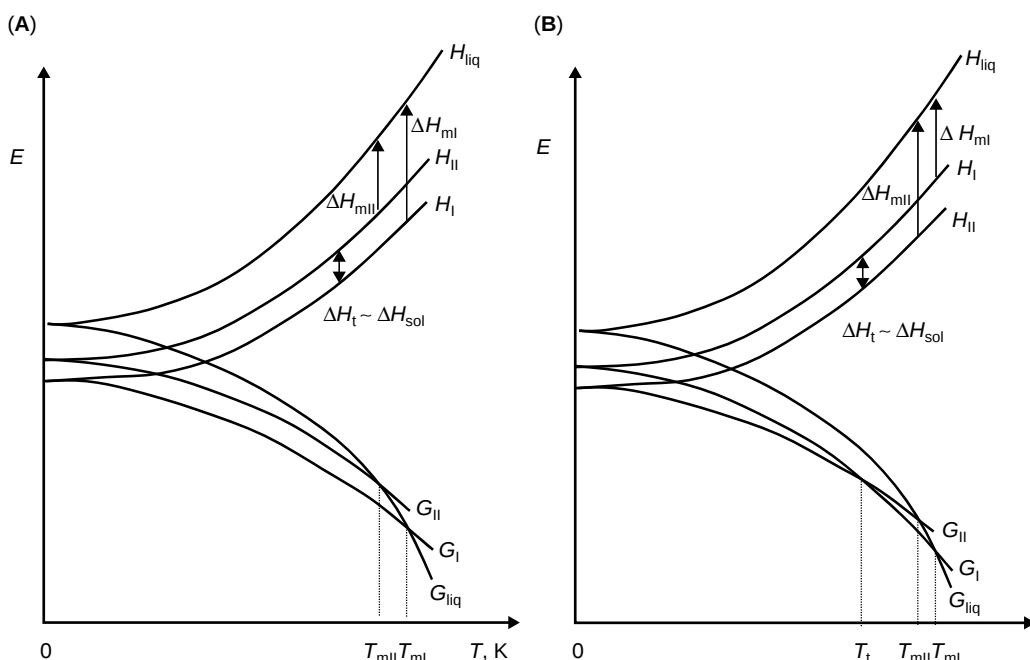


Figure 11 Semiquantitative energy-temperature diagrams for (A) monotropically and (B) enantiotropically related crystal polymorphs.

differences (ΔG) between polymorphs (107). Collectively, DSC, solubility, IDR and/or solution calorimetry data allow polymorph stability to be quantitatively studied over a wide range of temperatures. Practical aspects of assessing thermodynamic stability through a combination of DSC, slurry conversion and solubility data have been detailed by Threlfall (108).

Provided that the transformation kinetics between different neat and hydrated crystal forms are sufficiently rapid, moisture sorption isotherms can provide insights into thermodynamic RH stability relationships at a given temperature. In cases where conversion of an anhydrate into a hydrate (or of a lower hydrate into a higher hydrate) is rapidly reversible, the thermodynamic RH stability relationship will be readily apparent. If, on the other hand, the forms show appreciable kinetic stability over a range of humidities and either no conversion or hysteresis is observed in the moisture sorption isotherm, then the critical RH at which the thermodynamic stability order of the forms reverses will not be obvious. In these cases, slurry equilibration, which accelerates the slow transformation kinetics of crystals surrounded by vapor, can be used to determine the specific RH (water activity) at which the hydrate (or higher hydrate) becomes thermodynamically more stable (109). Slurry bridging, a variant of the slurry conversion experiment discussed previously, entails introducing two or more crystal forms in a suspension to overcome the nucleation kinetics of the more stable form. When conducted in aqueous-organic solvent mixtures wherein the water activity is systematically varied by changing the solvent composition, the direction of the phase transformation will reverse on either side of the critical water activity. Of course, slurry bridging is equally useful in assessing the thermodynamic stability of true polymorphs. To the extent that the dissolution and crystal growth kinetics are affected by the solubility of the drug substance, the outcome of slurry bridging true polymorphic forms will be independent of the solvent. In designing and interpreting the results of slurry bridging experiments, it is important to recognize that near the thermodynamic transition temperature of enantiotropic polymorphs or the critical water activity where the free energies of the forms converge, there will be no thermodynamic driving force for conversion to the more stable form.

Excipient Compatibility

Excipients are routinely used to enhance drug product performance, but when placed in intimate contact with drugs, they may also compromise it either directly by reacting with the drug substance or indirectly by providing a source of water and/or pH modification. Historically, excipient compatibility testing of pharmaceuticals has focused on chemical decomposition, the loss of potency due to formation of related substances, while physical stability testing has been relegated to the study of phase transitions in pure drug substances. Physical transformations augmented by excipients in a formulation may also adversely impact the chemical stability, dissolution performance and bioavailability of the drug product, but have received far less attention. Here, we look at physical transformations induced by water and excipients and their impact on chemical stability, as well as the influence that solid-state form can have on the compatibility of the drug substance with excipients.

Water, as discussed earlier, plays a major role in inducing both physical and chemical instability in pharmaceutical solids through a number of different mechanisms. When the humidity surrounding a solid exceeds a critical RH that is characteristic of a specific form of a given compound (RH_o), the solid will sorb moisture and dissolve within the water forming a solution until the activity of the water within the solution is equal to that in the vapor to which it is exposed. Leeson and Mattocks, in their study of aspirin decomposition, determined that degradation took place primarily in the saturated solution that had formed by continuous moisture sorption on the surface of particles (110). With deliquescent substances, the presence of another water-soluble substance, such as a more hydrolytic counterion, excipient or reaction product accumulated in the mixture, can drive the deliquescence process by changing the thermodynamics of the system and increasing the driving force for water condensation. Saleme and Taylor recently demonstrated the deliquescence lowering effect of citric acid on sucrose inversion (hydrolysis to glucose and fructose), showing that increased water sorption of sucrose–citric acid mixtures at RHs below RH_o caused solid dissolution and subsequently led to a pronounced increase in chemical reactivity (111). In such cases, where the critical humidity of the mixture, $RH_{o(mix)}$, is substantially lower than the RH_o of either deliquescent component, compounds need to be shielded from relative humidities that exceed not merely RH_o , but rather $RH_{o(mix)}$ to ensure their physical and chemical integrity.

In a closed system, hygroscopic excipients can function as dessicants, slowing moisture ingress into hygroscopic drug substances by competitively sorbing available moisture (112). Taylor et al. having examined the influence of a variety of excipients on the kinetics of hydration and dehydration, found that excipients generally promote dehydration and only occasionally promote hydration (113). Clearly, if phase stability is a potential issue for the drug substance, the influence of formulation excipients on the transformation kinetics should be carefully examined. Hygroscopic excipients must themselves be stable if they are to “protect” an active ingredient from moisture-induced phase transformations or chemical degradation. Herman et al. attributed the increased rate of hydrolysis of methylprednisolone sodium succinate in freeze-dried mannitol formulations to the migration of water into regions of the amorphous drug substance following crystallization of the excipient (114). Jain et al. showed that a leukotriene receptor antagonist mixed with lactose monohydrate was more resistant to degradation than when mixed with lactose anhydrous (115). In this case, the phase transition of lactose anhydrous to the hydrated form during wet granulation caused more intimate contact between the drug and excipient leading to decreased stability. These studies indicate that it is perhaps best if phase transitions of any kind are avoided during formulation or at a minimum their impact is fully understood.

Many reactions are directly influenced by the ionization state of the reacting functionality. In the case of peroxide oxidation, salt forms may be used to stabilize a compound by protonating reactive amine(s). Quite often, however, salts are placed in formulations where the micro-environment within the tablet or capsule has a pH which favors dissociation back to its free form. The impact of salt disproportionation on drug product performance was clearly evident for delavirdine mesylate, which disproportionated in tablets stored at high RH (116). Not only

did the lower solubility of the free base lead to poorer dissolution, but the methanesulfonic acid released from the salt reacted with the disintegrant, croscarmellose sodium, effectively compromising the tablet disintegration. Studies conducted to understand the pH microenvironment of solid dosage forms have shown that the surface acidity (or basicity) of solid excipients is influenced not only by the chemical nature of the excipient, but also sorbed water on the solid surface (117). When acid/base catalyzed degradation or salt disproportionation is a concern, the pH microenvironment may be controlled using pH modifiers that influence the pH of sorbed water on the solid surface around the drug.

Different approaches have been taken to examine the physical stability of solid pharmaceuticals and to evaluate their potential interactions with excipients. The compatibility of an active ingredient and individual excipients is commonly studied in binary blends as a function of temperature and RH over time. While potentially revealing specific interactions directly, this approach can generate a large number of samples and may neglect the multifactorial nature of drug instability in formulations. For instance, significant amounts of free water may be introduced into a formulation by one component, such as a disintegrant, while the reactive functionality or substance creating a pH microenvironment that leads to the instability might be introduced by another component. Mg stearate, a commonly used lubricant, shows little propensity for inducing disproportionation when simply mixed with water insoluble salts of weak bases; however, in Mg stearate blends containing other excipients which themselves contain significant amounts of water, the phase stability of the base salts is frequently compromised. In this case, Mg stearate does not react directly with the base salt. Instead, MgO, a by-product present in production lots of Mg stearate, reacts with water to form $\text{Mg}(\text{OH})_2$, effectively raising the pH microenvironment in the blend above the pH_{max} of the salt where disproportionation becomes favored (118).

Because multicomponent sources of drug instability in formulations cannot be identified using simple binary blends of the drug substance and individual excipients, we have found it more useful to examine the performance of drugs holistically in model formulations. To this end, we have introduced physical stability stress testing in prototype formulations commonly used in high shear wet granulation and roller compaction tablet and capsule manufacturing, as detailed in Table 3. The physical form stability studies, which are conducted in conjunction with chemical stability testing, entail loading prototype formulations with ~30% drug to ensure that solid-state transformations are readily detected by XRPD (or solid-state ^{13}C NMR spectroscopy) and stressing the mixtures for 1–4 weeks at 70°C/75% RH. If instability from a physical change that could compromise drug product performance (e.g., dissolution) or chemical reactivity is identified, then studies may be initiated to identify specific incompatibilities between the drug substance and but a few excipients. Although the primary objective of the physical stability testing is to establish the stability of solid-state forms in formulations with respect to phase transformations, for example, anhydrous forms to hydrated forms (and vice versa), conversion of one polymorphic form to another, and dissociation of salts into their crystalline neutral forms, the learning can potentially influence excipient and manufacturing platform selection.

Process-Induced Phase Transformations

Evaluating the solid-state chemistry of drug crystal forms and their chemical and physical compatibility with excipients, particularly in the presence of moisture, is critical, but not sufficient, for ensuring drug product performance. Common pharmaceutical processing steps themselves, such as granulation, drying, milling, and compaction, not only promote interactions between reactive components compromising chemical and physical stability, but they may also temporarily place them in regions of phase space (elevated temperatures, high pressures) where forms become metastable and susceptible to conversions to other forms, crystalline and amorphous (5). Although phase transformations mediated by water, temperature, pressure, sheer and/or excipients during pharmaceutical processing are well

Table 3 Placebo Blends Used in Stress Stability Studies

Component	Formula #1		Formula #2		Formula #3	
	(mg/cap)	(%w/w)	(mg/cap)	(%w/w)	(mg/cap)	(%w/w)
Lactose spray dried	51.0	42.5	—	—	—	—
Microcrystalline cellulose	51.1	42.5	51.8	43.125	—	—
Povidone	6.0	5.0	—	—	—	—
Croscarmellose sodium	9.1	7.5	—	—	—	—
Sodium lauryl sulfate	1.5	1.25	—	—	—	—
Magnesium stearate	1.5	1.25	1.5	1.25	—	—
Mannitol	—	—	51.8	43.125	—	—
Hydroxypropylcellulose	—	—	6.0	5.0	—	—
Low-substitution hydroxy propyl cellulose	—	—	9.1	7.5	—	—
Pregelatinized starch	—	—	—	—	105	87.5
Partially pregelatinized starch with 5% silicone	—	—	—	—	15	12.5
Final blend	120	100	120	100	120	100

documented, surprisingly few in-depth investigations into the thermodynamics and kinetics of process-induced phase transformations have been reported (119,120). Selecting the thermodynamically most stable form identified from solid-state form screening provides a useful starting point for mitigating the risk of undesired solid-state transformations during storage, provided the stable form at that temperature and RH has been identified. To ensure that the crystallization process selects for the preferred solid-state form and to understand the conditions under which undesired phase transformations become thermodynamically favorable during pharmaceutical processing, the entire phase diagram must be considered as well. Minimally, the stability of the process-relevant forms should be evaluated under the conditions (pressure, volume, temperature, water activity) encountered during processing. Finally, the solid-state characterization of materials should be conducted at various stages of pharmaceutical processing, when possible. A staged approach can be invaluable when it comes to tracing sources of chemical instability or poor dissolution performance, for example, when a drug substance forms a hydrate during wet massing in the granulation fluid, only to dehydrate to its “original” form during drying.

Low Level Detection of Physical Forms

The minimum requirements of chemical and physical purity to ensure the stability of a pharmaceutical product are case-dependent and impossible to define a priori. Trace chemical impurities have been credited in crystallization processes for the appearance or disappearance of crystalline forms, alteration of crystal size produced, or complete inhibition of crystallization (121–124). Seed crystals, sometimes present at levels undetectable using current methods, can overcome the energetic barriers to crystal nucleation facilitating phase transformations with fluctuations in temperature or humidity. Defect sites and amorphous phases can also, as discussed earlier, have a pronounced effect on the physical and chemical stability of the drug product. While sophisticated analytical methods are readily available to detect and quantify even trace chemical impurities, detecting and quantifying low levels of physical forms, crystalline and amorphous, present a significant challenge for most drug substances, not to mention drug products. Attempts have been made to establish limits of detection or quantification across a range of solid-state techniques and case studies have been reported wherein phase impurities were detected at levels as low as 0.5%, depending on the technique (121,125,126). Such low detection limits are, in the authors’ experience, more the exception than the rule,

however (94). Certainly, reports of detection limits no better than 10–20% are unlikely to make their way into the literature. What must be understood is that any method, solid-state or otherwise, will only be useful in cases where a direct correlation can be made between the impurity level that is detected by the method and that responsible for observed chemical or physical instability. For solid-state methods, it is not uncommon for low level phase impurities responsible for adversely impacting drug substance and drug product stability to go undetected even after significant method development.

CONCLUSION

The solid-state form of a drug can have a major effect on drug product quality, safety and performance. Crystalline forms are generally preferred for the isolation and purification of a drug substance, as well as for the exquisite control they confer over physicochemical properties. As the crystalline solid-state almost always imparts stability to drug compounds, rarely will chemical stability be a concern for drug substances that are stable in solution. Therefore, an understanding of the intrinsic chemical reactivity of a drug molecule in solution will in most cases, serve as a basis for evaluating reactivity in the solid state induced by changes in temperature, pressure, RH (water), pH, excipients or combinations thereof. Understanding the solid form landscape of a drug molecule, on the other hand, will serve as a framework for evaluating risks of physical transformations during pharmaceutical processing and throughout the shelf-life of the drug product. Changes in the physical form of the drug will not only lead to greater chemical reactivity for inherently unstable molecules, but they may also compromise drug product performance through changes in other properties, such as solubility or dissolution rate. Only through comprehensive salt (/cocrystal) and polymorph screening will the solid form landscape of a drug be sufficiently understood so as to ensure that a suitable solid-state form, one that is physically *and* chemically stable, is incorporated by design into a quality drug product.

REFERENCES

1. Pudipeddi M, Serajuddin ATM, Grant DJW. Salt-selection strategies, In: Stahl PH, Wermuth CG, eds., *Handbook of Pharmaceutical Salts: Properties, Selection, and Use*. Weinheim: Wiley-VCH, 2002: 19–39.
2. Gould PL. Salt selection for basic drugs. *Int J Pharm* 1986; 33: 201–17.
3. Hilfiker R, De Paul SM, Szelagiewicz M. Approaches to polymorphism screening. In: Hilfiker R, ed., *Polymorphism in the Pharmaceutical Industry*. Wiley VCH Verlag GmbH & Co. KGaA: Weinheim 2006: 287–308.
4. Byrn SR, Pfeiffer RR, Stowell JG, eds. Part 5: Chemical transformation in the solid-state. In: *Solid-State Chemistry of Drugs*. 2nd edn, West Lafayette, IN: SSCI Inc, 1999.
5. Zhang GZZ, Law D, Schmitt EA, Qiu Y. Phase transformation considerations during process development and manufacture of solid oral dosage forms. *Adv Drug Del Rev* 2004; 56: 371–90.
6. Dunitz JD, Gavezzotti A. Molecular recognition in organic crystals: directed intermolecular bonds or nonlocalized bonding? *Angew Chem Intl Ed* 2005; 44: 1766–87.
7. Bernstein J. Introduction and historical background. In: *Polymorphism in Molecular Crystals*. IUCr Monographs on Crystallography. Oxford: Oxford University Press, 2002: 1–9.
8. Gavezzotti A. A solid-state chemist's view of the crystal polymorphism of organic compounds. *J Pharm Sci* 2007; 96: 2232–41.
9. van de Streek J. Searching the cambridge structural database for the 'best' representative of each unique polymorph. *Acta Crystallogr B* 2006; 62: 567–79.
10. Paul IC, Curtin DY. Thermally induced organic reactions in the solid-state. *Acc Chem Res* 1973; 6: 217–25.
11. Pikal MJ, Lukes AL, Lang JE. Thermal decomposition of amorphous beta-lactam antibacterials. *J Pharm Sci* 1977; 66: 1312–16.
12. Byrn SR, Xu W, Newman AW. Chemical reactivity in solid-state pharmaceuticals: formulation implications. *Adv Drug Deliv Rev* 2001; 48: 115–36.

13. McBride, JM. The role of local stress in solid-state radical reactions. *Acc Chem Res* 1983; 16: 304–12.
14. McBride JM, Segmuller BE, Hollingsworth MD, Mills DE, Weber, BA. Mechanical stress and reactivity in organic solids. *Science* 1986; 234: 830–5.
15. Byrn SR, Sutton PA, Tobias B, Frye J, Main P. The crystal structure, solid-state NMR spectra, and oxygen reactivity of five crystal forms of prednisolone *tert*-butylacetate. *J Am Chem Soc* 1988; 110: 1609–14.
16. Moorthy JN, Venkatakrishnan P. Double [2+2] photocycloaddition: topochemical conversion of 4-methyl-7-styrylcoumarin dimorphs into a strained cyclophane. *Cryst Growth Des* 2007; 7: 713–18.
17. Ichikawa M, Takahashi M, Aoyagi S, Kibayashi C. Total synthesis of (–)-incarvilline, (+)-incarvine C, and (–)-incarvillateine. *J Am Chem Soc* 2004; 126: 16553–8.
18. Turowska-Tyrk I. Structural transformations in organic crystals during photochemical reactions. *J Phys Org Chem* 2004; 17: 837–47.
19. Chen X, Morris KR, Griesser UJ, Byrn SR, Stowell JG. Reactivity differences of indomethacin solid forms with ammonia gas. *J Am Chem Soc* 2002; 124: 15012–19.
20. Khawam A, Flanagan DR. Basics and applications of solid-state kinetics: a pharmaceutical perspective *J Pharm Sci* 2006; 95: 472–98.
21. Carstensen JT, Attarchi F, Hou XP. Decomposition of aspirin in the solid-state in the presence of limited amounts of moisture. *J Pharm Sci* 1985; 74: 741–5.
22. Ahlneck C, Zografi G. The molecular basis of moisture effects on the physical and chemical stability of drugs in the solid-state. *Int J Pharm* 1990; 62: 87–95.
23. Ball MC. Solid-state hydrolysis of aspirin. *J Chem Soc Faraday Trans* 1994; 90: 997–1001.
24. Guerrieri PP, Smith DT, Taylor LS. Phase behavior of ranitidine HCl in the presence of degradants and atmospheric moisture: impact on chemical stability. *Langmuir* 2008; 24: 3850–6.
25. Teraoka R, Otsuka M, Matsuda Y. Effects of temperature and relative-humidity on the solid-state chemical-stability of ranitidine hydrochloride. *J Pharm Sci* 1993; 82: 601–4.
26. Hancock BC, Parks M. What is the true solubility advantage for amorphous materials? *Pharm Res* 2000; 17: 397–404.
27. Good DJ, Rodríguez-Hornedo N. Solubility advantage of pharmaceutical cocrystals. *Cryst Growth Des* 2009; 9: 2252–64.
28. Pudipeddi M, Serajuddin ATM. Trends in solubility of polymorphs. *J Pharm Sci* 2005; 94: 929–39.
29. Kumar L, Amin A, Bansal AK. Salt selection in drug development. *Pharm Tech* 2008; 128–46.
30. Paulekuhn GS, Dressman JB, Saal C. Trends in active pharmaceutical ingredient salt selection based on analysis of the orange book database. *J Med Chem* 2007; 50: 6665–72.
31. Bastin RJ, Bowker MJ, Slater BJ Salt selection and optimization procedures for pharmaceutical new chemical entities. *Org Proc Res Dev* 2000; 4: 427–35.
32. Adeef A, Comer JEA, Thomson SJ. pH-metric log *P*. 3. Glass electrode calibration in methanol-water, applied to pKa determination of water-insoluble substances. *Anal Chem* 1993; 65: 42–9.
33. Anderson BD, Flora KP. Preparation of water-soluble compounds through salt formation. In: Wermuth CG ed., *The Practice of Medicinal Chemistry*. London: Academic, 1996: 739–54.
34. Serajuddin ATM. Salt formation to improve drug solubility. *Adv Drug Del Rev* 2007; 59: 603–16.
35. Streng WH, Hsi SK, Helms PE, Tan HG. General treatment of pH-solubility profiles of weak acids and bases and the effects of different salts on the solubility of a weak base. *J Pharm Sci* 1984; 73: 1679–84.
36. Bogardus JB, Blackwood RK. Solubility of doxycycline in aqueous solution. *J Pharm Sci* 1979; 68: 188–94.
37. Guerrieri P, Taylor LS. Role of salt and excipient properties on disproportionation in the solid-state. *Pharm Res* 2009; 26: 2015–26.
38. Berge SM, Bighley LD, Monkhouse DC. Pharmaceutical salts. *J Pharm Sci* 1977; 66: 1–19.
39. Ledwidge MT, Corrigan OI. Effects of surface active characteristics and solid-state forms on the pH solubility profiles of drug–salt systems. *Int J Pharm* 1998; 174: 187–200.
40. Childs SL, Stahly GP, Park A. The salt-cocrystal continuum: the influence of crystal structure on ionization state. *Mol Pharm* 2007; 4: 323–38.
41. Steiner T. The hydrogen bond in the solid-state. *Angew Chem Int Ed* 2002; 41: 48–76.
42. Stahly GP. Diversity in single- and multiple-component crystals. The search for and prevalence of polymorphs and cocrystals. *Cryst Growth Des* 2007; 7: 1007–26.
43. Trask AV, Motherwell WDS, Jones W. Pharmaceutical co-crystallization: engineering a remedy for caffeine hydration. *Cryst Growth Des* 2005; 5: 1013–21.

44. Trask AV, Motherwell WDS, Jones W. Physical stability enhancement of theophylline via cocrystallization. *Int J Pharm* 2006; 320: 114–23.
45. Bender DM, Bao J, Dantzig AH et al. Synthesis, crystallization and biological evaluation of an orally active prodrug of gemcitabine. *J Med Chem* 2009; 52: 6958–61.
46. Blagden N, de Matas M, Gavan PT, York P. Crystal engineering of active pharmaceutical ingredients to improve solubility and dissolution rates. *Adv Drug Del Rev* 2007; 59: 617–30.
47. Bethune SJ, Huang N, Jayansankar A, Rodríguez-Hornedo N. Understanding and predicting the effect of cocrystal components and pH on cocrystal solubility. *Cryst Growth Des* 2009; 9: 3976–88.
48. Nehm SJ, Rodríguez-Spong B, Rodríguez-Hornedo N. Phase solubility diagrams of cocrystals are explained by solubility product and solution complexation. *Cryst Growth Des* 2006; 6: 592–600.
49. Amos JG, Indelicato JM, Pasini CE, Reutzel SM. U.S. Patents 5,412,094 and 6,001,996.
50. Childs SL, Rodríguez-Hornedo N, Reddy LS et al. Screening strategies based on solubility and solution composition generate pharmaceutically acceptable cocrystals of carbamazepine. *CrystEngComm* 2008; 10: 856–64.
51. International conference on harmonization; guidance on Q6A specifications: test procedures and acceptance criteria for new drug substances and new drug products: chemical substances. *Fed Reg* 2000; 65: 83041–63.
52. Yu LX. Pharmaceutical quality by design: product and process development, understanding and control. *Pharm Res* 2008; 25: 781–91.
53. Price SL. The computational prediction of pharmaceutical crystal structures and polymorphism. *Adv Drug Deliv Rev* 2004; 56: 301–19.
54. Johnston A, Florence AJ, Shankland N et al. Crystallization and crystal energy landscape of hydrochlorothiazide. *Cryst Growth Des* 2007; 7: 705–12.
55. Price SL. From crystal structure prediction to polymorph prediction: interpreting the crystal energy landscape. *Phys Chem Chem Phys* 2008; 10: 1996–2009.
56. Price SL. Computed crystal energy landscapes for understanding and predicting organic crystal structures and polymorphism. *Acc Chem Res* 2009; 42: 117–26.
57. Neumann MA, Perrin MA. Can crystal structure prediction guide experimentalists to a new polymorph of paracetamol? *Cryst Eng Comm* 2009; 11: 2475–9.
58. McCrone WC. In: *Physics and Chemistry of the Organic Solid-State*. Vol 2. Fox D, Labes MM, Weissberger A (Eds.) Wiley Interscience, New York 1965; 726–67.
59. Cambridge Crystallographic Data Centre, Statistics. <http://www.ccdc.cam.ac.uk/products/csd/statistics/> (September 27, 2009)
60. Griesser U. In: *Polymorphism in the Pharmaceutical Industry*. Weinheim Germany: Wiley-VCH, 2006; 211–33.
61. Gu C-H, Young Jr V, Grant DJW. Polymorph screening: influence of solvents on the rate of solvent-mediated polymorphic transformation. *J Pharm Sci* 2001; 90: 1878–90.
62. Trask AV, van de Streek J, Motherwell SW, Jones W. Achieving polymorphic and stoichiometric diversity in cocrystal formation: importance of solid-state grinding, powder X-ray structure determination, and seeding. *Cryst Growth Des* 2005; 5: 2233–41.
63. Gu CH, Li H, Gandhi RB, Raghavan K. Group solvents by statistical analysis of solvent property parameters: implication to polymorph screening. *Int J Pharm* 2004; 283: 117–25.
64. Peresypkin A, Variankaval N, Ferlita R et al. Discovery of a stable molecular complex of an API with HCl: a long journey to a conventional salt. *J Pharm Sci* 2008; 97: 3721–6.
65. Giliyar C, Fickstad DT, Tyavanagimatt S. Challenges and opportunities in oral delivery of poorly-soluble drugs. *Drug Deliv Tech* 2006; 6: 57–63.
66. Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv Drug Deliv Rev* 2001; 46: 3–26.
67. Giovacazzo C, Monaco HL, Viterbo D et al. Physical properties of crystals. Chapter 9. *Fundamentals of Crystallography*, Oxford: Oxford Science Publications, 1995.
68. Trueblood KN, Buerger HB, Burzlaff H et al. Atomic displacement parameter nomenclature report of a subcommittee on atomic displacement parameter nomenclature. *Acta Cryst A* 1996; A52: 770–81.
69. Muller P. Practical suggestions for better crystal structures. *Cryst Reviews* 2009; 15: 57–83.
70. Feng T, Pinal R, Carvajal MT. Process induced disorder in crystalline materials: differentiating defective crystals from amorphous form of griseofulvin. *J Pharm Sci* 2008; 97: 3207–21.

71. Chang I, Fujara F, Geil B et al. Translational and rotational molecular motion in supercooled liquids studied by NMR and forced rayleigh scattering. *Non-Cryst Solids* 1994; 248: 172–4.
72. Mao C, Chamrathy SP, Byrn SR, Pinal RA. Calorimetric method to estimate molecular mobility of amorphous solids at relatively low temperatures. *Pharm Res* 2006; 23: 2269–76.
73. Johari GP, Goldstein MJ. Viscous liquids and the glass transition: II. Secondary relaxations in glasses of rigid molecules. *Chem Phys* 1970; 53: 2372–88.
74. Hatase M, Hanaya M, Hikima T, Oguni M. Discovery of homogeneous-nucleation-base crystallization in simple glass-forming liquid of toluene below its glass-transition temperature. *J Non-Cryst Solids* 2002; 307: 257–63.
75. Sun Y, Xi H, Chen S, Ediger MD, Yu L. Crystallization near glass transition: transition from diffusion-controlled to diffusionless crystal growth studied with seven polymorphs. *J Phys Chem B* 2008; 112: 5594–601.
76. Gutzow I. In: Induced crystallization of glass forming system: a case of transient heterogeneous nucleation Part I; *Contemporary Physics Volume 21* (2); 1980.
77. Hancock B, Zografi G. Characteristics and significance of the amorphous state in pharmaceutical systems *J Pharm Sci* 1997; 86: 1–12.
78. Shamblin SL, Tang X, Chang L, Hancock BC, Pikal MJ. Characterization of the time scales of molecular motion in pharmaceutically important glasses. *J Phys Chem B* 1999; 103: 4113–21.
79. Bhugra C, Pikal MJ. Role of thermodynamic, molecular, and kinetic factors in crystallization from the amorphous state. *J Pharm Sci* 2008; 97: 1329–49.
80. Yu L. Amorphous pharmaceutical solids: preparation, characterization and stabilization. *Adv Drug Del Rev* 2001; 48: 27–42.
81. Hancock BC, Shamblin SL, Zografi G. Molecular mobility of amorphous pharmaceutical solids below their glass transition temperatures. *Pharm Res* 1995; 12: 799–806.
82. Makower B, and Dye WB. Sugar crystallization equilibrium moisture content and crystallization of amorphous sucrose and glucose. *Agricultural and Food Chem* 1956; 4: 72–7.
83. Imaizumi H, Nambu N, Nagai T. Stability and several physical properties of amorphous and crystalline form of indomethacin. *Chem Pharm Bull* 1980; 28: 2565–9.
84. Adronis V, Yoshioka M, Zografi G. Effects of sorbed water on the crystallization of indomethacin from the amorphous state. *J Pharm Sci* 1997; 86: 346–51.
85. Simha R, Boyer RF. On a general relation involving the glass temperature and coefficients of polymers. *J Chem Phys* 1962; 37: 1003–7.
86. Levine H, Slade L. Water as a plasticizer: physicochemical aspects of low-moisture polymeric systems. In: Franks, F. ed. *Water Science Reviews*, Cambridge: Cambridge University Press, 1987: 79–185.
87. Matsumoto T, Zografi G. Physical properties of solid molecular dispersions of indomethacin with poly(vinylpyrrolidone) and poly(vinylpyrrolidone-co-vinylacetate) in relation to indomethacin crystallization. *Pharm Res* 1999; 16: 1722–7.
88. Brenker MJ, Schneider HA, Cantow HJ. Approach to the composition dependence of the glass transition temperature of compatible polymer blends. *Polymer* 1988; 29: 78–85.
89. Zheng X, Yang R, Zhang Y. Bioavailability in beagle dogs of nimodipine solid-dispersions prepared by hot-melt extrusion. *Drug Dev Ind Ph* 2007; 33: 783–9.
90. Sekiguchi K, Obi N, Ueda Y. Studies on absorption of eutectic mixture: I. A comparison of the behavior of eutectic mixture of sulfathiazole and that of ordinary sulfathiazole in man. *Chem Pharm Bull (Tokyo)* 1961; 9: 866–72.
91. Chiou WL, Niazi S. Phase diagram and dissolution rate studies on sulfathiazole-urea solid dispersions. *J Pharm Sci* 1971; 60: 1333–8.
92. Hancock BC, Zografi G. The relationship between the glass-transition temperature and the water-content of amorphous pharmaceutical solids. *Pharm Res* 1994; 11: 471–7.
93. Chow K, Tong HHY, Lum S, Chow AHL. Engineering of pharmaceutical materials: an industrial perspective. *J Pharm Sci* 2008; 97: 2855–77.
94. Stephenson GA, Forbes RA, Reutzel-Edens SM. Characterization of the solid-state: quantitative issues. *Adv Drug Del Rev* 2001; 48: 67–90.
95. Saleki-Gerhardt A, Ahlneck C, Zografi G. Assessment of disorder in crystalline solids. *Int J Pharm* 1994; 101: 237–47.
96. Bates S, Zografi G, Morris K, Crowley K, Newman A. Analysis of amorphous and nanocrystalline solids from their X-ray diffraction patterns. *Pharm Res* 2006; 23: 2333–49.

97. Clinical studies, which will serve as the basis for claims in the product label (e.g., efficacy, drug-drug-interactions, food effect studies, etc.), are considered "pivotal".
98. Chemburkar SR, Bauer J, Deming K et al. Dealing with the impact of ritonavir polymorphs on the late stages of bulk drug process development. *Org Proc Res Dev* 2000; 4: 413–17.
99. Reutzel-Edens SM. Achieving polymorph selectivity in the crystallization of pharmaceutical solids: basic considerations and recent advances. *Curr Opin Drug Disc Dev.* 2006; 9: 806–15.
100. Threlfall T. Crystallization of polymorphs: thermodynamic insight into the role of solvent. *Org Process Res Dev* 2000; 4: 384–90.
101. Rodriguez-Hornedo N, Murphy D. Significance of controlling crystallization mechanisms and kinetics in pharmaceutical systems. *J Pharm Sci* 1999; 88: 651–60.
102. Morris KR, Fakes MG, Thakur AB et al. An integrated approach to the selection of optimal salt form for a new drug candidate. *Int J Pharm* 1994; 105: 209–17.
103. Newman AW, Reutzel-Edens SM, Zografi G. Characterization of the "hygroscopic" properties of active pharmaceutical ingredients. *J Pharm Sci* 2008; 97: 1047–59.
104. Yu L, Reutzel SM, Stephenson GA. Physical characterization of polymorphic drugs: an integrated characterization strategy. *Pharm Sci Technol Today* 1998; 1: 18–27.
105. Burger A, Ramberger R. On the polymorphism of pharmaceuticals and other molecular crystals: I. Theory of thermodynamic rules. *Mikrochim Acta* 1979; 259–71.
106. Grunenberg A, Henck J-O, Siesler HW. Theoretical derivation and practical application of energy/temperature diagrams as an instrument in preformulation studies of polymorphic drug substances. *Int J Pharm* 1996; 129: 147–58.
107. Yu L. Inferring thermodynamic stability relationship of polymorphs from melting data. *J Pharm Sci* 1995; 84: 966–74.
108. Threlfall TL. Turning DSC charts of polymorphs into phase diagrams: a tutorial paper. *Org Proc Res Dev* 2009; 13: 1224–30.
109. Ticehurst MD, Storey RA, Watt C. Application of slurry bridging experiments at controlled water activities to predict the solid-state conversion between anhydrous and hydrated forms using theophylline as model drug. *Int J Pharm* 2002; 247: 1–10.
110. Leeson LJ, Mattocks AM. Decomposition of aspirin in the solid-state. *J Am Pharm Assoc* 1958; 47: 329–33.
111. Salameh AK, Taylor LS. Role of deliquescence lowering in enhancing chemical reactivity in physical mixtures. *J Phys Chem B* 2006; 110: 10190–6.
112. Kesavan JG, Peck GE. Solid-state stability of theophylline anhydrous in theophylline anhydrous-polyvinylpyrrolidone physical mixtures. *Drug Dev Ind Pharm* 1996; 22: 189–99.
113. Salameh AK, Taylor LS. Physical stability of crystal hydrates and their anhydrides in the presence of excipients. *J Pharm Sci* 2006; 95: 446–61.
114. Herman BD, Sinclair BD, Milton N, Nail SL. The effect of bulking agent on the solid-state stability of freeze-dried methylprednisolone sodium succinate. *Pharm Res* 1994; 11: 1467–73.
115. Jain R, Railkar AS, Malick AW, Rhodes CT, Shah NH. Stability of a hydrophobic drug in presence of hydrous and anhydrous lactose. *Eur J Pharm Biopharm* 1998; 46: 177–82.
116. Rohrs BR, Thamann TJ, Gao P et al. Tablet dissolution affected by a moisture mediated solid-state interaction between drug and disintegrant. *Pharm Res* 1999; 16: 1850–6.
117. Govindarajan R, Zinchuk A, Hancock B, Shalaev E, Suryanarayanan R. Ionization states in the microenvironment of solid dosage forms: effect of formulation variables and processing. *Pharm Res* 2006; 23: 2454–268.
118. Remington In: *The Science and Practice of Pharmacy* 21st edn. Philadelphia: Lippincott Williams and Wilkins, 2006: 108.7.
119. Morris KR, Griesser UJ, Eckhardt CJ, Stowell JG. Theroetical approaches to physical transformations of active pharmaceutical ingredients during manufacturing processes. *Adv Drug Del Rev* 2001; 48: 91–114.
120. Wang J, Davidovich M, Desai D et al. Solid-state interactions of a drug substance and excipients and their impact on tablet dissolution: a thermal-mechanical facilitated process-induced transformation or PIT. *J Pharm Sci* 2010; 99: 3849–62.
121. Dunitz JD, Bernstein J. Disappearing polymorphs. *Acc Chem Res* 1995; 28: 193–200.
122. Davey RJ, Blagden, N, Potts GD, Docherty R. Polymorphism in molecular crystals: stabilization of a metastable form by conformational mimicry. *J Am Chem Soc* 1997; 119: 1767–72.

123. Wikstrom H, Rantanen J, Gift AD, Taylor, LS. Toward an understanding of the factors influencing anhydrate-to-hydrate transformation kinetics in aqueous environments. *Cryst Growth Des* 2008; 8: 2684–93.
124. Bauer J, Spanton S, Henry R et al. Ritonavir: an extraordinary example of conformational polymorphism. *Pharm Res* 2001;18: 859–66.
125. Shah B, Kakumanu K, Bansal A. Analytical techniques for quantification of amorphous/crystalline phases in pharmaceutical solids. *J Pharm Sci* 2006; 95: 1641–65.
126. Lehto VP, Tenho M, Vaehae-Heikkilae K et al. The comparison of seven different methods to quantify the amorphous content of spray dried lactose. *Powder Tech* 2006;167: 85–93.

11 | Solid-state excipient compatibility testing

Amy S. Antipas, Margaret S. Landis, and W. Peter Wuelfing

Selection of appropriate excipients for formulation of drug substances is vitally important in ensuring final product stability and efficacy (1). As formulation scientists, it is imperative that we develop pharmaceutical products that have acceptable chemical and physical stability during the time period of their distribution, storage, and use. It is not uncommon that an API (active pharmaceutical ingredient) will be stable as bulk drug but unstable when blended with the excipients required for formulation of dosage forms. Therefore, understanding the reactivity of the API in the solid state when mixed with excipients is critical to commercial formulation development.

Since the gathering of real-time stability and compatibility data is impractical in the early stages of development, scientists must rely on accelerated stability testing to develop a fundamental understanding of the chemical functionality of excipients and stability methods for predicting ambient condition interactions and degradation rates. Often, such studies will require innovative approaches to minimize the amount of API needed and maximize the detection of small quantities of degradation products. The application of the holistic understanding of reactivity of both API and excipients early in and throughout development is consistent with the increased interest in “quality by design” (QbD) formulation approaches. The rational design of formulations (QbD) starts in the preformulation space, where the first guiding data become available to steer product formulation and development. These data are critical in identifying major risks with an API early in the formulation design space, enabling development organizations to minimize (or eliminate) delays due to surprise instability issues as drug candidates move closer to regulatory filing. This chapter will focus on summarizing approaches for designing, measuring, predicting, and interpreting solid-state excipient compatibility data.

DESIGNS OF TRADITIONAL EXCIPIENT COMPATIBILITY EXPERIMENTS

Attributes of API Preformulation Profiles

Prior to the initiation of any solid-state excipient compatibility testing of a potential drug candidate, it is best to generate a preformulation profile of the API (2,3). This profile should include pH-solubility profiles, pH-stability profiles, pK_a determination, and generation of log P information, as well as knowledge about degradation products formed in the solution state under acidic, basic, oxidative, and oxidative/free radical stress conditions (4). Corroboration of this preformulation stability data in relation to a detailed examination of the chemical structure, where nucleophilic, electrophilic, hydrogen-bond donating, and hydrogen-bond accepting sites are identified, gives the solid dosage formulator potential insight into the areas of molecular reactivity of the API with excipients during formulation studies (5). If available, it is always worthwhile to review the metabolites associated with in vivo degradation of the API, as it can yield important information on reactive areas of the molecular scaffold. Additional information on the molecular reactivity can also be predicted by available computer models (6,7).

While solution-state properties and stability data are important, it is ultimately the characteristics of the solid state of the API that are most important to the solid-state formulator. Therefore, it is recommended that a critical mass of information on the solid-state form of the API be gathered prior to initiation of any compatibility studies. Relevant solid-state parameters of the API that could be investigated for further information include (but are not limited to):

- Thermal and thermal/humidity stability
- Hygroscopicity
- Thermal calorimetric behavior
- Single crystal or crystal packing information

- Particle-size distribution and surface area
- Crystal habit and/or amorphous content
- Hot-stage polarized light microscopy data
- Photostability
- Effects of mechanical aggravation

Minimally, one should have a brief foreknowledge of the thermal and thermal/humidity solid-state stability of the API prior to initiation of excipient compatibility studies (8). These protocols should include the investigation of stability at various temperature and humidity conditions and should generate information about both the chemical stability and physical form integrity of the API. Thermal and thermal/moisture induced solid-state chemical reactions are well known (9,10), with hydrolysis and oxidation being the most prevalent mechanisms of decay. Changes in the physical form with thermal and moisture stress are also common and well described in the literature (11,12). Published reviews (13) describe many standard spectroscopic techniques used to characterize physical form changes of the bulk API.

Another important parameter to investigate prior to excipient studies is the ability of the compound to gain or lose water when exposed to variable humidity environments, as it is known that water will equilibrate and redistribute rapidly in solid-state mixtures. Solid state photochemical assessment should be considered as it can be dramatically different than solution state photoreactivity (14) and a set of brief, simple studies can provide early warnings for preparation, storage and subdivision lighting precautions.

Thermal analysis can provide much information about the solid state API (15) for consideration during the planning of excipient compatibility testing. Melting point and heat of fusion data provide an understanding of the bonding strengths within the crystal lattice. Thermal transitions associated with a particular API form can be observed and recorded to detail thermal polymorphic interconversion, dehydration/desolvation, and decomposition temperatures. Knowledge of temperature-related behavior of the API should guide storage and evaluation methods for the excipient compatibility studies and circumvent the use of incompatible storage and testing conditions.

There are a number of solid-state aspects of the API that should be briefly examined prior to commencement of compatibility studies. Knowledge of crystal habit, particle size distribution, and surface area are important because these characteristics will affect the stability of solid dosage forms in excipient compatibility testing (16,17). While single crystal data may not be available at early formulation stages, predicted data may be available, based on powder x-ray diffraction studies, and should be considered (18,19).

During tablet production, the API will be subjected to high mechanical stress from processes like high-shear blending, mechanical granulation and compaction (20). Assessment of the effects of mechanical aggravation on the API alone and the inclusion of grinding and compaction aspects in preparation of materials for excipient compatibility studies will provide a more realistic view of stability observed in larger scale production in later stages of development. Crystalline to amorphous transformations and polymorphic transitions (21) are the most common changes in physical form associated with mechanical aggravation. Several examples of perturbation in chemical stability due to mechanical activation are known. It has been shown (22) that increased amorphous content can be correlated to increased chemical reactivity of the API in the solid state. Waltersson and Lungren (23) investigated the increase in hydrolytic stability of model compounds (salicylic acid and procaine) following ball-milling and attributed the increased reactivity to the increase in surface activity and crystal defect sites. The tendency of an API to experience this type of activation is only recently beginning to be predictable (24); thus, some actual assessment of the propensity for mechanically induced changes should be evaluated prior to (or concomitant with) excipient compatibility studies. A variety of auxiliary methods have been described to assess these changes, such as changes in thermal calorimetry profiles (25), changes in vapor sorption (26), sensitive solution calorimetry (27–29), PXRD (30,31), optical microscopy, and solubility/dissolution characteristics (32). If conversion to the amorphous state is likely, it is

reasonable to gain an estimate of the rate of degradation under stability conditions. Amorphous material samples can be generated by various techniques for chemical stability studies including solvent cast films or collection of thin films evaporated from solvent in a rotary evaporator.

The overall purity of the API should be considered prior to investigation of excipient compatibility, such as starting impurity levels, levels of residual water and residual solvents, as well as the presence and levels of any heavy metal impurities. This consideration includes chiral purity (enantiomeric purity) and diastereomeric impurities of the material.

In general, if an API form has stability problems in the bulk form, it is best to solve these issues via salt and form selection studies designed to discover and identify more thermodynamically stable forms prior to any excipient compatibility evaluation. It is known that different polymorphic forms and hydrated/solvated forms can have dramatically different stability profiles (33–37).

Attributes of Excipients

Information about excipients is critical in the initial planning and interpretation of the excipient compatibility results. Important factors to consider for excipients include their physical and chemical properties. The *Handbook of Pharmaceutical Excipients* lists important information on structure, moisture content, melting point, pH, solubility, and equilibrium moisture at variable relative humidity (38). The properties of excipients should be evaluated considering the modes of degradation seen in solution state stress testing studies with the API. An example of relevant physical and chemical parameters for some select excipients is detailed in Table 1. A spectroscopic review of excipients (39) has been completed and extensive reviews of some of the most common types of excipients (i.e., carbohydrate based) are published (40).

Two key parameters of excipients that are important to the compatibility formulator are (i) the ability of the excipients to absorb water at variable humidity and (ii) the pH the excipient will impart in the solid-state aka “microenvironmental pH” (41). Knowledge of water sorption of most excipients is well documented (38) and is used to estimate the amount of water the excipients will introduce into a solid formulation mixture (42). If the excipients in question are not characterized, they can be evaluated using simple vapor sorption experiments by the investigator. The percentage of water and the dispensation of water, whether bound or variably adsorbed, was shown to have a dramatic effect on the hydrolytic stability of aspirin (43). It is well known that water will redistribute within solid pharmaceutical systems and this

Table 1 Relevant Physical–Chemical Properties of Some Selected Common Tablet Excipients

Excipient (Common Use)	Moisture Content	Melting Point	pH	Solubility	Equilibrium Moisture at 75% RH
Microcrystalline cellulose (diluent)	Typically less than 5% w/w	260–270°C (decomp)	5.0–7.0	Practically insoluble in water	6% w/w gain
Lactose monohydrate (diluent)	4.5–5.5% w/w (5% as monohydrate water)	201–202°C	5.0–7.0	Soluble	1% w/w gain
Calcium phosphate dibasic dihydrate (diluent)	21% w/w as dihydrate water	Decomposes above 100°C with loss of water	7.4	Practically insoluble in water	19%w/w
Sodium starch glycolate (disintegrant)	As much as 10% w/w	200°C (decomp)	5.5–7.5	Practically insoluble in water	23% w/w gain
Ascorbic acid (antioxidant)	0.1% w/w	190°C (decomp)	2.1–2.6	Soluble	–

redistribution has been modeled and studied extensively (26). The pH influence that an excipient imparts to the formulation matrix can be directly related to chemical stability. Because many excipients are generally insoluble in water, a standard method to assess excipient pH is measurement of 5–20% w/w slurries in water. If preformulation studies have determined that pH plays a major role in degradation of an API, excipients with compatible pH profiles can be selected. For example, the degradation of a fluoropyridinyl drug in a capsule formulation was correlated with the pH of the excipients and not with the amount of moisture present (44).

Other key solid-state experimental information useful to gather on the excipients include:

- known incompatibilities of excipients
- known stabilization effects of excipients
- reactive impurities in excipients
- mechanical properties of the excipients

One should consider known chemical incompatibilities of excipients with specific classes of API containing certain functional groups. Known incompatibilities are detailed and well documented in standard references (5,38). The most well-known example of incompatibility is reducing cellulosic excipients with primary and secondary amine containing drugs, which is commonly referred to as Maillard-type degradation (45–47). Based solely on initial examination of the chemical structure of the API, many carbohydrate excipients can be immediately ruled out of testing protocols.

On the other hand, many excipients can act to chemically stabilize an API in the solid state and in solid dosage forms. A common class of stabilizing excipients is cyclodextrins (48,49). Cyclodextrins can envelop the API in their hydrophobic cavities and shield it from common degradation reactions such as hydrolysis, oxidation, or photodegradation. Other excipients or additives may also act as complexing agents that provide hydrolytic (50) and oxidative (51) stabilization. Many excipients, dyes and colored additives are capable of providing extensive photostabilization in the solid state (52–55). For lyophilized formulations certain polymers act as stabilizers by limiting the mobility of water in the solid state (56).

A brief review of the excipient synthesis and isolation can give vital clues about the presence of potential reactive impurities remaining from processing and derivatization reactions. Examples include the known residual presence of sodium glycolate in sodium glycolate, a common tablet disintegrant, that can play a role in chemical instability of the API (38). In another study, authors describe how a low level lactose impurity, lactose-phosphate, was responsible for the accelerated degradation of a steroid compound by driving generation of an enol intermediate (57). Known impurities of enteric polymers, such as hydroxypropyl methylcellulose acetate succinate and hydroxypropyl methylcellulose phthalate, have been identified as sources of degradation of duloxetine hydrochloride in solid dosage formulations (58).

Wasylaschuk et al. (59) and others (60,61) have quantitated trace level peroxide content in many common soluble and insoluble excipients, including PEGs of multiple molecular weights, polysorbate 20 and polysorbate 80, hydroxypropyl cellulose, polyvinylpyrrolidone (PVP), and lactose. In these papers, methods are presented that report either as nmol peroxide per gram of excipient or ppm levels, both of which significantly lower the limit of detection versus compendial methods. In the Wasylaschuk paper, values reported for excipients range from less than 10 to greater than 5000 nmol peroxide/g, with a large range noted across multiple batches analyzed. For instance, the common excipient hydroxypropyl cellulose (HPC) revealed a range of 100 to 900 nmol peroxide/g depending on age and vendor which could be a root cause of stability variability if a compound is sensitive to peroxide driven degradation routes. Table 2 summarizes the excipient characterization data across the papers referenced and is consistent in showing increasing impurity level with increasing ethoxylation, unsaturation, and liquid physical state. Tracking these impurities at trace levels as well as understanding lot-to-lot variability allows the formulation scientist to

Table 2 Peroxide Levels in Pharmaceutical Excipients

Excipient	Number of Lots Tested	Mean Peroxide (nmol/g)	Range (nmol/g)
Lactose ^a	5	<10	<10–10
HPC ^a	21	300	50–890
PEG400 ^a	4	2,200	1,000–3,300
Polysorbate 80 ^a	8	1,500	180–4,600
PVP ^a	5	7,300	3,600–11,000
Microcrystalline cellulose ^a	5	<10	<10–10
PEG solid (3400, 4600, and 6000) ^a	4	20	<10–40
Poloxamer (188,338,407) ^a	7	30	10–50
Mannitol ^a	5	<10	All lots <10
Sucrose ^a	5	<10	<10–20
Povidone ^b	1	124 ppm	NA
Crospovidone ^b	1	130 ppm	NA
Crospovidone ^c	6	33 ppm	22–45 ppm

^aAdapted from Ref. 59.^bAdapted from Ref. 60.^cAdapted from Ref. 61.

better understand the extent and likely reproducibility of chemical degradation of API in formulations. Furthermore, the levels of excipient peroxide levels can increase under formulation processing conditions, and thus control of process parameters to ensure suitable control of peroxide generation may also need to be considered (62). Degradation routes of concern include nucleophilic reactions of drug with peroxides yielding *N*-oxides or sulfoxides as well as the autoxidation of drug initiated by peroxy radicals generated from excipient peroxide breakdown. A simple calculation highlights the need for these enhanced detection limits. Consider a hypothetical soft gelatin capsule formulation containing 10 mg of a 300 g/mol molecular weight drug substance. If this compound was formulated with 500 mg of an excipient containing 1000 nmol peroxide/g, the result would be a drug:peroxide molar ratio of ~ 67:1 or 1.4% in the capsule. This level would be alarming if the compound is shown to be reactive with peroxides given typical ICH degradation identification and qualification limits of around 0.1% versus active. The distribution of organic hydroperoxide versus hydrogen peroxide content is discussed in Wasylaschuk et al. (59) and results show the distribution is linked to the excipient synthetic route as well as possible degradation of peroxides in excipients like PEG400.

Following observations of oxidative degradation of raloxifene hydrochloride in tablets and direct mixtures containing povidone and crospovidone excipients, Hartauer et al. (60) proposed that peroxide impurities in the povidone and crospovidone were directly responsible for the degradation observed. Accelerated binary studies of the API with all the individual tablet components revealed that povidone and crospovidone were the most likely cause of the oxidative instability (Table 3).

To further elaborate on this degradation, studies involving the addition of hydrogen peroxide directly to tablet formulation matrices of raloxifene hydrochloride prior to granulation and compaction were examined. Stability results again supported the indictment of peroxide impurities in the povidone and crospovidone excipients used in the core tablet formulation (Fig. 1) as being responsible for oxidation of raloxifene to the *N*-oxide degradation product. Increasing amounts of added peroxide led directly to increasing amounts of the *N*-oxide degradation product being formed in the tablet formulations. These peroxide spiking experiments helped in the full identification of the *N*-oxide degradation product and provided valuable data

Table 3 N-Oxide Levels for Drug Excipient Mixtures After 31 days Storage at 125°C (N-Oxide Expressed as % w/w of Raloxifene-HCl)

Drug-Excipient Mixture	Amount (n=1)
R-HCl/lactose anhydrous	0.01
R-HCl/lactose monohydrate	0.01
R-HCl/povidone	0.26
R-HCl/croscopovidone	0.26
R-HCl/magnesium stearate	0.01
R-HCl/polysorbate 80	0.03

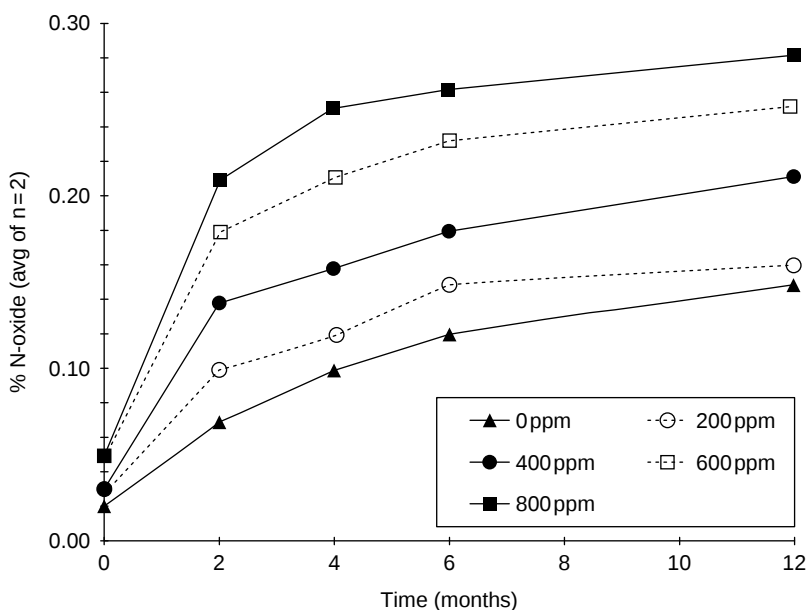


Figure 1 Influence of peroxide impurities on Raloxifene chemical stability.

for a realistic control strategy that would impose limits on the impurities allowed in designated excipients for manufacture of the final dosage form product.

Peroxides continue to degrade further into reactive aldehyde impurities that can degrade drug substances as well. Li et al. have characterized the aldehyde content with a GC method selective for different aldehydes and quantitated the formaldehyde and acetaldehyde content in most commonly used excipients (63). The values ranged from 0.5 to greater than 10 ppm across the solid and liquid excipients and values trend directly with peroxide content as expected and are listed in Table 4. Li et al. continued their work quantifying aldehyde growth under ICH storage conditions where formaldehyde content in PEG400 tripled from ~100 to 300 ppm at 40°C/75%RH storage for 8 weeks (64).

Review of the mechanical properties of the excipients is important when initiating excipient compatibility studies. These parameters are well studied for many excipients and their importance in solid dosage form design and compatibility has been extensively reviewed (65,66). If excipient compatibility studies will involve mechanical processing and compacting

Table 4 Aldehyde Content in Common Excipients as Measured by Gas Chromatography

Excipient	Aldehydes Detected ($\mu\text{g/g}$)	
	Formaldehyde	Acetaldehyde
Butylated hydroxyanisole	N.D.	N.D.
Corn oil	N.D.	N.D.
Crospovidone	2.4	4.5
Dibasic calcium phosphate	N.D.	N.D.
Hydroxypropyl cellulose	0.6	0.5
Lactose	0.1	N.D.
Microcrystalline cellulose	0.4	0.1
Mono- and di-glycerides	N.D.	N.D.
Poloxamer 407	0.1	2.0
PEG400	>10 ^a	8.2
PEG600	>10 ^a	4.1
PEG4000	0.4	2.1
Povidone	4.7	4.2
Mannitol	0.2	N.D.
Lactose	0.1	N.D.
Sucrose	5	<10

^aExceeded method linearity.

Abbreviation: N.D., Not detected.

of samples, knowledge of these material parameters is very useful. Compaction pressure leading to physical form transitions (67) may affect the API stability in excipient mixtures and tablets (68,69) and the degree of interaction of the API with all components and impurities of the excipient mixtures. In addition of changes in crystal habit, the production of amorphous content in an API with processing is known (70). Increased amorphous content can lead to a higher degree of molecular mobility and diffusion in the formulation matrix, leading to a higher degree of accessibility of the API to formulation impurities (peroxides, trace metals, aldehydes, etc.) The grade of excipient material used in the compatibility studies should be similar to that used in later stage solid dosage form development for accurate prediction of compatibility.

Any and all preliminary information on the solid-state form and degradation susceptibility of the API and characteristics of the excipients will enable the scientist to (i) design the best experimental layout for excipient compatibility studies that focuses on the relevant aspects of the API and excipients (ii) have a better understanding of appropriate stress conditions and timeframes over which to conduct the studies, and (iii) provide a more complete basis for which to interpret the final results of the excipient compatibility testing.

Initiation of Excipient Compatibility: Design of Experiments

According to Monkhouse (71), there are four major attributes of a drug-excipient compatibility study: (i) sample preparation, (ii) statistical design, (iii) storage conditions, and (iv) method of analysis. Traditionally, a one factor at a time approach employing the preparation of binary blends of the API with excipients has been used in compatibility screening. Many different ratios of API to excipient have been reported, but one should attempt to reflect the relative amounts in a tablet in order to obtain the most meaningful data. Blends can be prepared by dry mixing the components in a vial using a vortex; however, be aware that the homogeneity

Table 5 Box–Wilson-Type Fractional Factorial Design

Trial Number	Factors						
	X_1	X_2	X_3	$X_4 (X_1X_2)$	$X_5 (X_1X_3)$	$X_6 (X_1X_2X_3)$	X_2X_3
1	–	–	–	+	+	–	+
2	+	–	–	–	–	+	+
3	–	+	–	–	+	+	–
4	+	+	–	+	–	–	–
5	–	–	+	+	–	+	–
6	+	–	+	–	+	–	–
7	–	+	+	–	–	–	+
8	+	+	+	+	+	+	+

Abbreviations: X_1 , Lactose (–) or mannitol (+); X_2 , Stearic acid (–) or magnesium stearate (+); X_3 , PVP (–) or gelatin (+); X_4 , Maize starch (–) or Avicel (+); X_5 , Presence or absence (–) of light (+); X_6 , 0 (–) or 3 (+) % humidity.

of the blend will be important in ensuring that all of the API is in contact with the excipient and is especially critical if you are not sampling the entire vial. It should be noted that this process does not include any mechanical processing (mechanical blending and compaction), so the resulting stability may not be reflective of samples produced using larger scale production processes.

It has been reported that the chemical stability of a drug substance in a binary drug substance–excipient mixture may differ significantly or even completely from a multicomponent drug substance–excipient mixture because of the potential for interactions to occur between the excipients as well as between the drug and excipients (72). A well-conceived statistical design could easily reveal these additional interactions. According to the proponents of statistical designs, more information can be obtained with less work, iteration time and cost versus the one factor at a time approach. Some examples of statistical designs can be found in the following paragraphs.

El-Banna et al. described the use of a Box–Wilson type fractional factorial design (73). They explored six factors which included filler, lubricant, binder, disintegrant, light, and humidity. For the full factorial design ($N = 2^6$), six factors at two levels, 64 experiments must be performed. A more practical fractional factorial design ($N = 2^{6-3}$) containing eight experiments can still elucidate the main effects as well as some of the interactive effects as seen in the design in Table 5.

Durig and Fassihi utilized a Plackett–Burman saturated factorial design that allows accurate investigation of multiple factors simultaneously without having to investigate all possible combinations (74). Taken to the extreme, this type of design can be used to evaluate up to $N-1$ factors from only N experiments. One disadvantage of this approach is that there is no estimation of the inherent experimental error or measurement of the significance of observed effects because of the absence of replicates. Also, only the main effects are detected and not the interactions between two or more factors. As a result, the scientist must balance the significance and utility of the data versus the need to incorporate additional experiments. In examining the stability of pyridoxal HCl, Durig et al. employed a Plackett–Burman design consisting of 13 variables at two levels. As seen in Table 6, 24 experiments were used to examine the 13 variables, with the addition of pseudo variables, to help in determining the system error. Using the average effects of the pseudo variables, the standard deviation of the variable effect was calculated in order to provide estimations for the minimum significant variable effects at the 90% and 60% confidence levels. In this design, it was possible to observe both stabilizing and destabilizing effects of excipients.

Table 6 Plackett–Burman Design for Pyridoxal HCl Compatibility Studies [In the Table (+) Denotes High Level and (–) Denotes Low Level]

Trial	Variable												Pseudo Variable											
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
19	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
21	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
22	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
23	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	

Abbreviations: A, Stearic acid; B, Magnesium stearate; C, Aerosil 380; D, Lactose; E, Ludipress; F, Corn starch; G, Avicel PH101; H, Methycellulose; I, Ethylcellulose; J, Eudragit RSPM; K, Mannitol; L, Relative humidity; M, Temperature; N–W, Pseudo variables.

APPROPRIATE STRESS CONDITIONS

Thermal

Elevated thermal conditions are often used to accelerate chemical degradation processes that will occur at room temperature upon storage. In order to minimize the time required for the stress experiments, the experimental temperatures are often higher than 25°C. Care must be taken when using this method of stress since raising the temperature above 70–80°C may cause the energy of the system to exceed the activation energies of alternative and additional degradation mechanisms, thus resulting in unrepresentative data (75). Sims et al. studied the compatibility of SB-243213-A in excipient mixtures to explore conditions, which would have predicted the degradation that was seen with the clinical formulation after three months of storage at 50°C (76). As seen in Figure 2, when 100°C was used as the stress condition, the desired degradation products (Imp A and Im B) are formed but the additional peaks early in the chromatogram indicate a shift in the primary degradation processes. When the experimental temperature is lowered to 60°C and 80°C, the degradation profile is similar to what was expected based on ambient stability studies of the clinical tablet. Additionally, one will need to consider the melting point and glass transition temperatures of all excipients to ensure the chosen thermal stress conditions are appropriate to maintain the physical integrity of the sample components.

Mechanical Stress

Mechanical stress may be introduced into the system in several ways. Some of the most common include grinding or milling the drug or blend. By adding mechanical stress using these methods, it is hypothesized that amorphous pockets of drug are formed or crystal defects are induced such that the rate of degradation will increase. In studying the effects of mechanical comminution on the stability of procaine penicillin, Waltersson and Lundgren found that the rate of solid state degradation nearly doubled after the drug was ball milled for 20 hours, Table 7 (23). This trend continued when the drug was blended with Avicel® or Emcompress®.

Mechanical stress may also be added to the system through the use of compaction. Some possible reasons for differences in reactivity after compaction include the following:

- more intimate interactions of the drug with the excipients or impurities
- mechanically induced chemical reactions

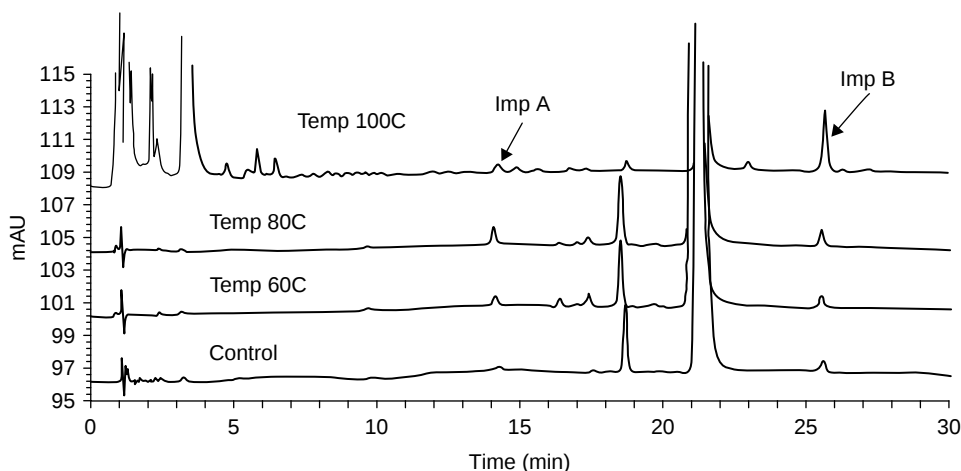
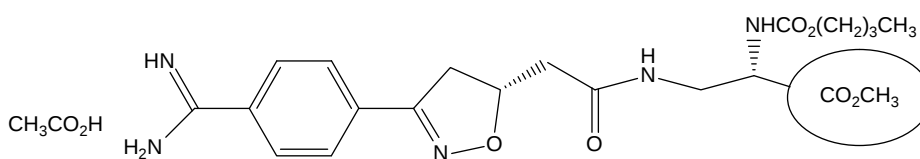


Figure 2 Degradation of SB-243213-A at different temperatures.

Table 7 Observed Rate Constant for the Solid-state Degradation of Procaine Penicillin G Stored at 55°C and 67% RH. [Excerpted from Table 3 in Waltersson and Lundgren (23)]

Sample	Rate Constant, k_0 (mg/day) (mean $\pm$ SD)
Pure drug, unmilled	0.240 $\pm$ 0.005
Pure drug, milled 20 hr	0.434 $\pm$ 0.011
Avicel® + unmilled drug	0.473 $\pm$ 0.012
Avicel® + milled drug	1.018 $\pm$ 0.008
Emcompress® + unmilled drug	0.428 $\pm$ 0.009
Emcompress® + milled drug	0.708 $\pm$ 0.013

**Figure 3** Structure of DMP-754. Area of ester hydrolysis is highlighted.**Table 8** Ester Hydrolysis of DMP-754 in Lactose Blends and Compacts after 1 month at 40°C/75% RH. [Values Extracted from Figure 3 in Badway et al. (77)]

	Hydrolysis (%)
Blend	1.75
Compact	6

- changes in physical form, that is, induction of amorphous defects, changes to partially amorphous or other physical forms
- different surface area of contact, that is, changes in surface area to volume ratio

In trying to formulate DMP-754, Badway et al. investigated binary blends as well as compacts of DMP-754 (Fig. 3) with anhydrous lactose. He concluded that compaction of their blends resulted in an increase in the amount of hydrolysis seen (Table 8) (77). They postulated that this result was due to an increase in the number of contact points between the drug and the lactose resulting in an increased rate of moisture transfer leading to degradation.

Guo et al. studied the relationship between the solid-state chemical instability of quinapril hydrochloride (QHCl, Fig. 4) and its physical characteristics (78). QHCl degrades via an intramolecular cyclization resulting in aminolysis and production of a diketopiperazine compound (DKP). Crystalline QHCl exists as an acetonitrile solvate, which is loosely incorporated into the lattice channels. As seen in Figure 5A and 5B, loss of the acetonitrile occurs with minimal mechanical force and results in loss of crystallinity. This desolvation event could result in an increase in the amount of degradation since the activation energy for the cyclization reaction is now significantly lower in the amorphous state than the crystalline state (Table 9) (79,80).

Compression of samples on a Carver press resulted in partial loss of crystallinity (Fig. 6) leading them to extrapolate that the compression force used in tableting could result in the formation of some amorphous character, which would decrease stability.

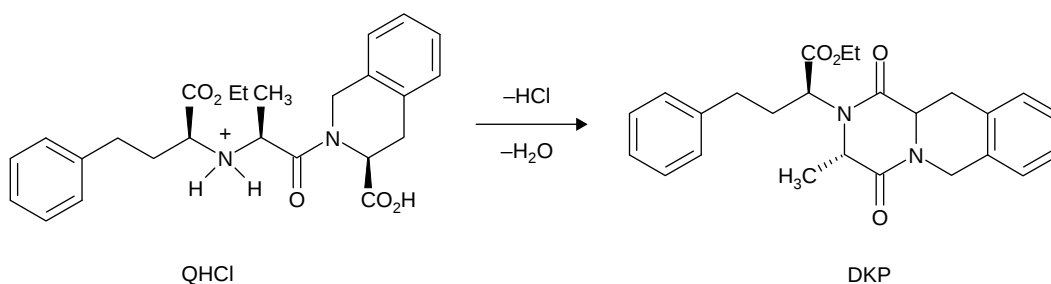


Figure 4 Structure of quinapril HCl (QHCl) and degradation to DKP.

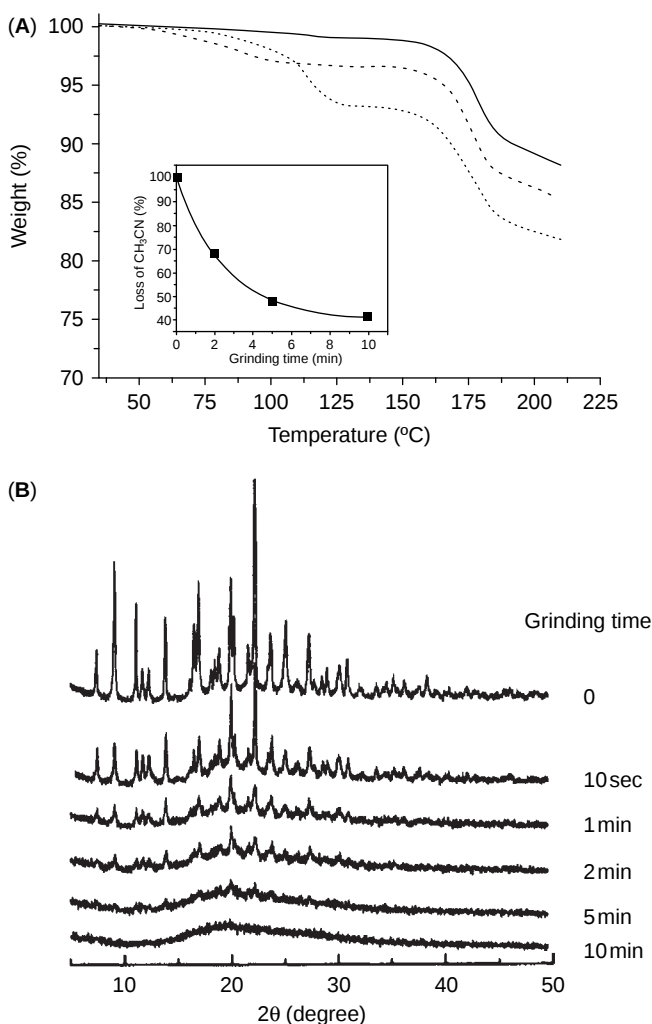
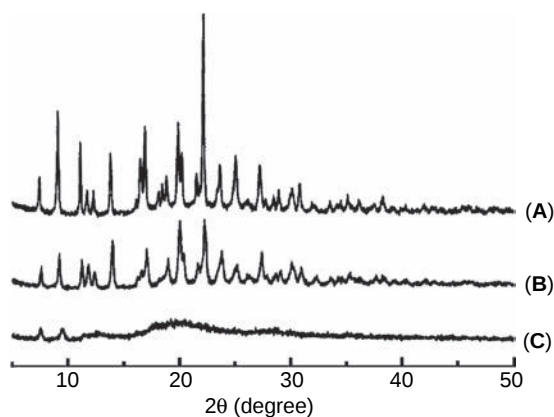


Figure 5 (A) TGA analysis of QHCl-CH₃CN describing percent weight loss with increasing temperature and the loss of acetonitrile in QHCl-CH₃CN versus grinding time (refer to inset). (B) PXRD patterns of samples obtained after grinding crystalline QHCl-CH₃CN for different time intervals.

Table 9 Comparison of Reactivity for the Cyclization Reaction of Amorphous QHCl to Similar Cyclization Reactions in Crystalline Solid (79) and Solution (80).

System	E_a (kcal/mol)
Crystalline	~60
Amorphous	30–35
In solution	20

**Figure 6** (A) PXRD of crystalline QHCl-CH₃CN; (B) after compression using a Carver press (~10⁴ lb/cm²); (C) sample after desolvation under vacuum at 45°C for 24 hours.

Compaction may decrease the reaction rate of compounds that degrade via a mechanism that produces a gaseous product, since the compression can serve to inhibit vapor escape. For example, in the quinapril HCl case, the degradation produces two gaseous products, HCl and water (78). Experiments exploring the effect of sample size indicate that the degradation rate is decreased when the sample size is large (Fig. 7). The results indicate that the restriction of gases to escape from the reaction matrix tends to slow down the reaction significantly. Compaction of the samples would be expected to have similar effects as larger samples. The reaction rate is increased when the gas removal is facilitated by applying a vacuum (inset in Fig. 7).

Polizzi proposed the generation of mechanoradicals during a high shear mixing process with microcrystalline cellulose to describe the approximately equal short term chemical degradation of both crystalline and amorphous CP-448,187 (81). The paper unambiguously describes an example that pharmaceutical processing, in this case a mechanical aggravation, can be a source of reactive impurities that ignite drug degradation. Masayuki describes via ESR a plausible route of radical formation (82), suggesting that cellulose undergoes 1,4-glucosidic bond cleavage and then hydrogen abstraction to form hydroperoxides. This reaction is then followed by the typical autoxidation processes that lead to drug oxidation in formulation (83) as shown in Figure 8.

Water

Water is often added into excipient compatibility designs since hydrolysis is one of the most common routes of drug degradation. Most excipients contain an appreciable level of free water or are able to absorb water from the environment (see section on "Attributes of Excipients"). Water can

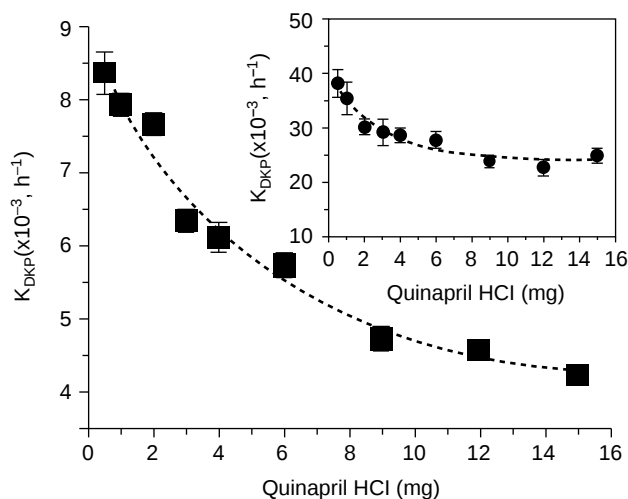


Figure 7 Effect of sample mass on the cyclization reaction rate constant of amorphous QHCl at 80°C. Inset shows results of same experiment but conducted under vacuum.

be added into the system through several methods: (i) the formation of a slurry or suspension, (ii) the addition of a certain percentage of water into a closed system, or (iii) exposure of the system to controlled humidity. The suspension technique can be used to rapidly assess whether chemical or physical stability problems exist; however, the presence of such an excess of water may cause a shift in degradation mechanisms from that seen in the solid state where the amount of water present depends on the properties of the drug and the excipients (84). Serajuddin et al. studied the degradation of multicomponent blends with various drugs after the addition of 20% water into a closed system (85). A specified amount of water was added into the system since their experience with humidity chambers was that the excipient interactions at high humidity depended on the amount of free moisture present and on the relative hygroscopicities of the drug substance and/or the excipients thus leading to variability in the data. In developing methods for on-line stress testing using a STEM block reactor, Sims et al. discovered that having a controlled and humid atmosphere produced more predictive results than having the sample in direct contact with the moisture (76). More traditional methods of excipient compatibility testing have employed the use of exposure to controlled humidity using saturated salt solutions or humidity chambers. The conditions used (-15°C , 5°C /ambient humidity, $25^{\circ}\text{C}/60\%\text{RH}$, $30^{\circ}\text{C}/60\%\text{RH}$, and $40^{\circ}\text{C}/75\%\text{RH}$) are often based on the guidance from the FDA entitled "Stability Testing of Drug Substances and Drug Products" (86).

Oxidation

Oxidative instability is the second most common cause of chemical degradation of API in pharmaceutical formulations. If prior knowledge and/or preformulation stability experiments have predicted reactivity toward oxidative degradation, additional oxidative stress conditions can be included into excipient compatibility protocols in several ways.

One of the most common ways to investigate the oxidative reactivity of compounds is to store the excipient blends in an environment having a pure oxygen headspace. It is generally accepted that acceleration of oxidation via this method may require high temperature and humidity (87). For these stress configurations to be accomplished, hermetically sealed containers, such as sealed glass septum vials or heat sealed foil packs and blisters, are highly recommended (88).

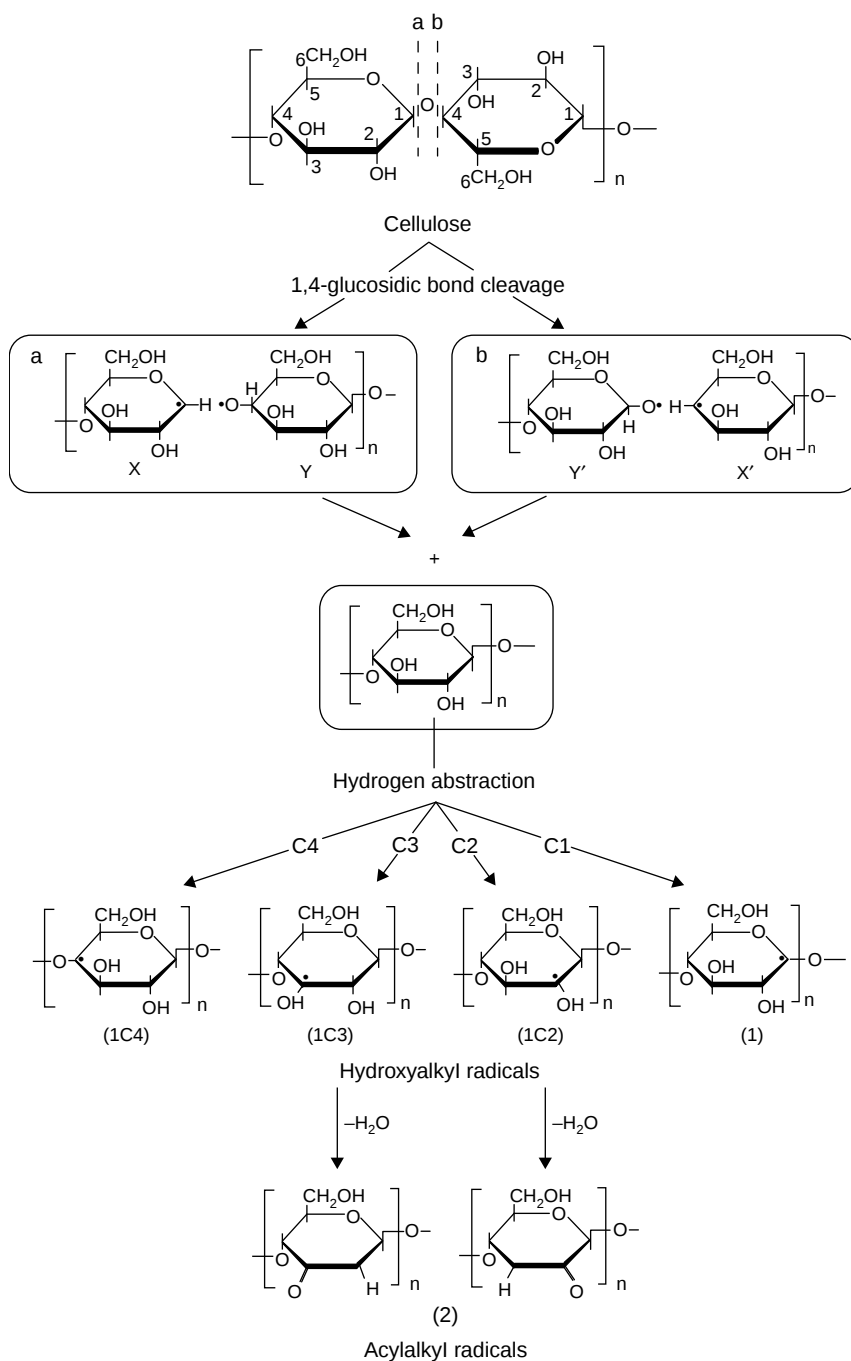


Figure 8 Proposed mechanolysis scheme for glucose-based polymers reprinted with permission from Masayuki et al. (82). Copyright 1999 American Chemical Society.

Harsher conditions may employ pressurized oxygen headspaces; for which Parr® type apparatus are employed. Incorporation of added metal impurities (commonly copper and iron salts) as low as submicromolar (89) in conjunction with oxygen headspace has been reported (90). One research group utilized high temperatures (80°C), copper (II) salts and oxygen gas headspace to study the decomposition of sulpyrine, noting that the oxidative decomposition depended heavily on water content (91). Not as common, but potentially still useful, is the use of radical initiators incorporated into solid dosage forms to accelerate oxidation rates. These samples can be stressed at increased temperature with oxygen headspaces (90).

For any gaseous headspace stress condition, particle size, and surface area may play a major role; thus, blends and compacts may show widely different results, with compacted material being the most realistic scenario for oxidation in solid dosage forms. Negative controls for this type of oxidative stress may involve the use of nitrogen (90) or argon headspaces. For further investigation into the mechanism and/or attempting to stabilize solid formulations toward oxidation, the formulator could use technologies such as oxygen scavengers (92) included in packaging configurations, less oxygen permeable capsule shells and tablet film coatings (93) having limited gas permeability.

As described previously in this chapter, common sources of oxidation in solid dosage forms are from excipient related peroxide impurities such as in polyvinylpyrrolidone (PVP) and low molecular weight polyethylene glycols (PEGs). These values are known to increase with age and open-air storage conditions; therefore, storage and handling history can be related to the levels of impurities and variation in the amount of oxidative degradation in compatibility studies and early solid dosage form evaluations.

Packaging Aspects

If the aim of excipient compatibility is to be predictive of the degradation expected with clinical or commercial dosage forms then the packaging configuration should be representative of what will be used in that setting. Factors that could influence stability measurements include container headspace as well as permeability of oxygen and water vapor through the walls and cap (if a bottle) of the container. Table 10 lists the water vapor transmission rates through common types of packaging materials (94).

As indicated by Waterman et al., the amount of water vapor permeating a typical HDPE bottle can be calculated using the following equation:

$$\text{Amount of permeating H}_2\text{O} = \frac{(\text{transmission rate})(\text{surface area of container})(\text{time})}{(\text{thickness of barrier})}$$

If hydrolysis is expected to be an issue, desiccants can be a valuable addition to the packaging scheme. The rate and capacity for water adsorption of desiccants will vary depending on

Table 10 Water Vapor Transmission Rates of Some Common Packaging Materials

Material	Water Vapor Transmission [g mm/(m ² day) at 38°C and 90% RH]
PVC	1.8
Polypropylene	0.54
HDPE	0.12
Aclar UltRx	0.006
Aclar 22A	0.011
Nylon 6	7.5–7.9
Oriented PET	0.39–0.51
Cold formed foil blister	<0.005

Table 11 Desiccant Options for Use with Pharmaceutical Products

Desiccant Type	g H ₂ O Adsorbed/g Adsorbent (25°C/75% RH)	g H ₂ O Adsorbed/g Adsorbent (25°C/20% RH)	g H ₂ O Adsorbed/g Adsorbent (25°C/10% RH)	Hrs to ½ Capacity (25°C/75% RH)	Approx. % RH Reached
Silica gel	0.33	0.12	0.05	1.2	10–20
Clay	0.26	0.11	0.08	1.5	10–20
Molecular sieves	0.22	0.18	0.15	0.5	2–10
CaSO ₄	0.10	0.05	0.03	1.2	15–30
CaO	0.28	0.28	0.27	27	1–25

Table 12 Oxygen Permeability (Transmission Rate) for Packaging Materials

Material	Oxygen Transmission [cm ³ mm/(m ² day atm)]
Low-density polyethylene	241
High-density polyethylene	102 (26.3 at 0°C)
Polypropylene	89
Polystyrene	127
Polyvinylchloride	4
Polycarbonate	114
Unoriented PET	2.5
Oriented PET	2

Abbreviation: PET, Polyethylene terephthalate.

the type of adsorbent and the configuration (i.e., canister versus sachet). Table 11 summarizes various desiccant options for use with pharmaceutical products (94).

Understanding oxygen transmission through commonly used pharmaceutical packaging materials (Table 12) will aid in the proper choice for stabilizing oxygen sensitive formulations (90). It should be noted that oxygen permeability of material often increases with temperature. The amount of oxygen permeating a typical HDPE bottle can be calculated in a similar manner to the method used for water vapor permeation.

A foil/foil blister sealed under a nitrogen atmosphere would provide the most protective packaging although this type of configuration can be expensive. An alternative would be the use of oxygen scavenging packets, which could be added to a bottle like traditional desiccant canisters. A packet of size 20 is able to scavenge up to 20 mL of O₂ or the equivalent of 100 mL of air (92). These oxygen scavengers are useful in stabilization of the product as well as diagnosis of the issue.

ANALYSIS

The initial analysis of excipient compatibility samples usually involves visual inspection of the samples for changes in color, tablet integrity, and deliquescence. Initial observations of this type can be a sign of instability of the API and excipients and should be noted and considered when assessing compatibility. Several subjective systems exist to quantify the changes in color following compatibility and stress testing of solid dosage forms (95). More recently, electronic systems are used to record, evaluate, and track the changes in tablet color (96,97) to avoid human subjectivity in assessments.

Measurement of Chemical Instability

HPLC

One of the most widely used methods for detecting the formation of degradation products after stability challenges in the face of excipients is the use of HPLC. Although the use of this technique requires sample preparation to extract the API and its degradants from the excipient

matrix, it remains a valuable tool since it has the ability to discriminate between decay products based on polarity. The ability to use various types of detectors (UV, MS, light scattering, charged aerosol detection, electrochemical, fluorescence, chemiluminescence) imparts flexibility and sensitivity to the technique. One essential requirement in the use of HPLC for the detection of degradation products is that the analytical method used must be stability indicating. In other words, the method must be capable of discriminating between the API and any decomposition products formed as well as be sufficiently sensitive to detect and quantify the degradation products (98). Since the identities of the degradation products are typically unknown at the time of early excipient compatibility studies, degradation is measured by comparing the relative peak areas of compounds eluting at specific retention times over some specified study duration. As mentioned by Olsen and Baertschi (99), it is important to use highly discriminating investigational methods capable of resolving and detecting degradation products so that mass balance can be assessed (100).

The combination of solid-phase extraction (SPE) with HPLC analysis or preparative HPLC can be valuable tools in concentrating and identifying degradation products (101). SPE can be a useful technique for the isolation and concentration of analytes from a complex mixture. Selection of the appropriate column depends on the properties of the API and the suspected degradation products (102,103). Mixed mode columns having both non-polar and ion exchange character can be useful in both the isolation and structural elucidation of degradation products. Examples of the use of solid phase extraction to characterize degradation in several pharmaceutical drug systems has recently been demonstrated (104).

Preparative HPLC is another convenient method for isolating degradation products from excipient compatibility matrices (105,106). The peaks from stressed samples can be collected, the solvent removed with a rotary evaporator and the remaining solution lyophilized to obtain purified compounds. The samples can then be analyzed by other methods such as mass spectrometry and NMR in order to identify the molecular composition.

Isothermal Microcalorimetry

Isothermal microcalorimetry has been used in the assessment of excipient compatibility in the solution and the solid state (27,107–109). As discussed by Buckton (29), the basis for evaluation of chemical interactions using this method is the measurement of minute amounts of evolved or absorbed heat after establishing a baseline at a constant temperature. The advantage of using this technique in the evaluation of excipient compatibility is that the data can be generated in the timeframe of several hours to a week as opposed to the traditional accelerated temperature methods that can require 6–12 weeks. Also, the sensitivity of the method may allow one to monitor the reaction at ambient temperatures rather than at highly elevated temperatures. One disadvantage of the technique is the non-specificity of the measurement. The evolution or absorption of heat could be a result of other physical processes occurring during the measurement such as dissolution, evaporation, phase transitions or crystallization (110). As a result, it is necessary to investigate the origins of the observed thermal events using additional assays. Since this technique is nondestructive, the sample is readily available to be analyzed by other techniques, such as HPLC, once the thermal measurement is complete (111).

Experimental procedures for running excipient compatibility studies using isothermal microcalorimetry include the collection of power-time curves for each component of the mixture alone, as well as in combinations (Fig. 9). The separate drug and excipient curves can then be used to construct a theoretical “noninteraction” curve for the blend, which then is subtracted from the actual blend curves in order to define the interaction between the components.

Variables that the investigator must keep in mind in designing isothermal microcalorimetry excipient compatibility studies are the effects of sample and excipient particle size, the drug to excipient ratio, the influence of different mixing methods, analysis temperature, and the effect of moisture on the samples. In general, for early studies, in order to maximize drug excipient interactions, the particle size of the drug and excipient are kept close as possible, the

ratio of drug to excipient used is 1:1 and the samples are run with high water content, either as added aliquots of water to the samples or after storage of the samples in a high controlled humidity chamber (i.e., 75% RH) prior to analysis. Selzer et al. has investigated the compatibility of a model drug in binary excipient mixtures (studied as powders, granules and tablets) (112,113). The data indicates that granulation or compaction of the powder results in a significantly higher heat flow as compared to the blend itself and is likely caused by the additional processing. This could be a result of increased association of the drug with the excipients or of physical processes occurring after compaction, such as relaxation.

Phipps et al (114) and Schmitt (110) have advocated using isothermal microcalorimetry as a screening tool to assign relative risk and eliminate highly unstable excipients from subsequent model formulations. As seen in Table 13, Schmitt found that the results of an excipient screen with ABT-627 using isothermal microcalorimetry at 50°C qualitatively agreed with

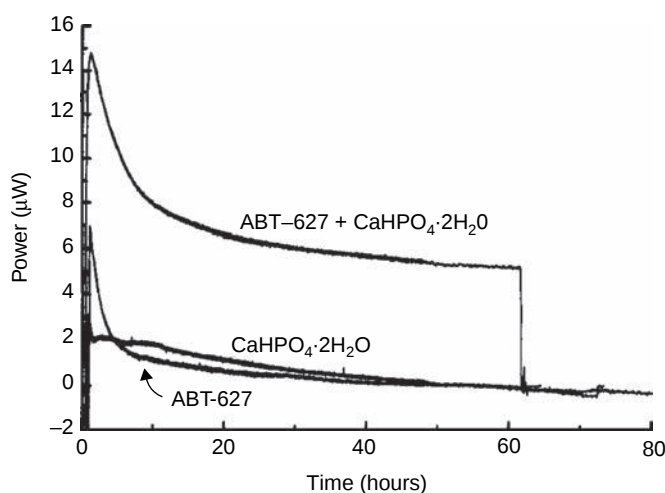


Figure 9 Typical raw power–time curves used for an excipient compatibility study.

Table 13 ABT-627 Excipient Compatibility Results Comparison Using Isothermal Microcalorimetry and HPLC Analysis of Binary Mixtures at 50°C [Extracted from Tables 1 and 2 in Schmitt et al. (110)]

Excipient	Microcalorimetry Interaction Power (Microwatts)	Area Percent Impurities by HPLC		
		Initial	After 3 wk	After 5.2 wk
Dibasic calcium phosphate	5.23	0	0.06	0.22
Microcrystalline cellulose	2.92	0	0.056	0.108
Lactose monohydrate	−0.345	0	0.051	0.076
Pregelatinized starch	−1.31	0.05	0.048	0.102
Magnesium stearate	6.51	0.546	1.25	2.04
Stearic acid	0.512	0	0.05	0.03
Sodium starch glycolate	7.12	0	0.21	0.51
Povidone K30	2.59	0.059	0.10	0.54
Crospovidone	2.37	0.052	0.25	0.39

the results obtained after the same blends were analyzed by HPLC after 3–6 weeks in a 50°C oven. The time to complete the excipient screening using microcalorimetry was significantly shorter than using conventional thermal methods (3 days vs. 6 weeks). This technique therefore may be a rapid, “fit for purpose” screening method for early excipient compatibility investigations.

High Sensitivity DSC (HS-DSC)

HS-DSC can be used as a screening tool to identify gross incompatibilities with excipients to recommend desired and undesired components of a possible test formulation (115,116). As with traditional DSC, a power differential is measured between a reference and the sample as a function of temperature. Changes in the melting behavior of the components in an excipient mixture, after comparison to those that occur with the individual components under the identical stress conditions, may be used as an indication of chemical incompatibility that occurs when the components are mixed. As indicated by Wissing et al. (117), the use of HS-DSC is controversial since melting differences may arise due to reasons other than chemical incompatibility. Initial studies from Mroso et al. (118), proclaiming the utility of this method, were run using a system where the incompatibility was linked to the melting behavior. It is debatable whether incompatibility between components of systems where the reaction is not linked to melting behavior could be detected (15).

Wissing (117) and McDaid (119), however, promoted the use of HS-DSC with stepwise isothermal ramping methods. Using ramping alone, the technique would have inferior sensitivity to microcalorimetry and using isothermal methods alone at room temperature may result in inferior sensitivity if the degradation mechanism is energetically weak. McDaid compared binary blends and compacts containing 5% API with 95% magnesium stearate or 95% lactose using HS-DSC to traditional accelerated methods where the samples were held for 6 weeks at 5°C/50% RH, 40°C/20% RH and 40°C/75% RH. HS-DSC samples were tested as the dry powder/compacts or as 2:1 slurry of water with solid. Temperature ramps ran from 30 to 90°C with the temperature held constant for 1–2 hours every 5°C (Figs. 10 and 11).

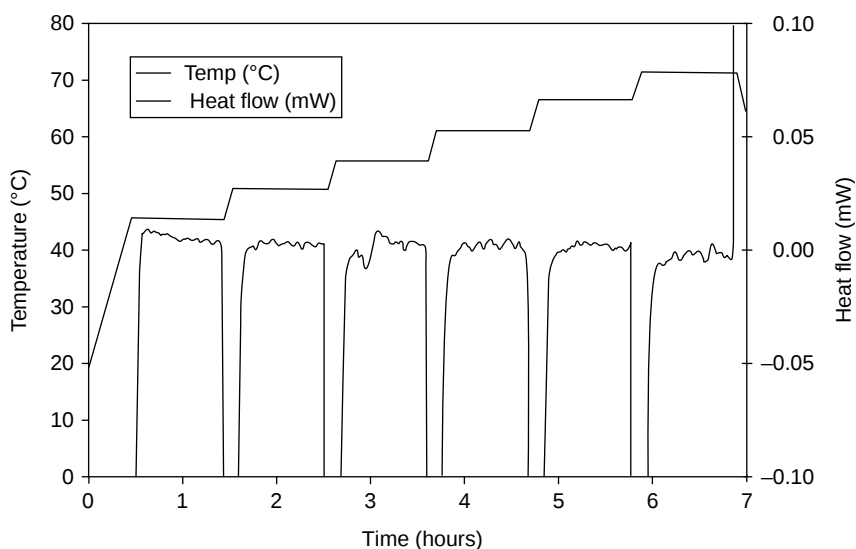


Figure 10 HS-DSC profiles for dry compacts of drug A and magnesium stearate (50/50; w/w) held for 1 hour every 5°C, total sample size ~75 mg.

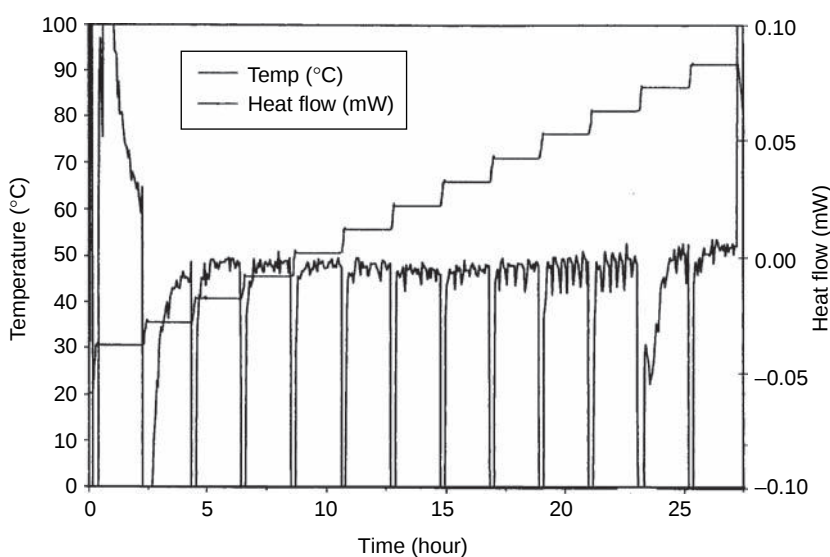


Figure 11 HS-DSC profiles for drug A-magnesium stearate-water slurries (16.7:16.7:66.6%; w/w), total sample size ~120 mg, showing thermal events at 35°C and 85°C (held for 2 hour every 5°C).

The results from the conventional excipient compatibility studies indicated an incompatibility of the API with magnesium stearate only at conditions of 40°C/75% RH. HS-DSC results indicated no incompatibilities in the dry state; however, an incompatibility with magnesium stearate was observed in the slurry. It was postulated that the thermal event at 35°C was due to dissolution of the drug into the aqueous phase of the slurry. This deviation was reduced when the sample was given sufficient time to dissolve before the experiment was initiated. A new thermal technique involving the use of localized thermomechanical analysis (L-TMA) and localized differential thermal analysis (L-DTA) to study drug-excipient compatibility at a particulate levels offers advantages over HS-DSC, such as much smaller sample size and more information about the physical nature of the drug-excipient samples and their interactions (120).

Physical Form Stability and Assessment

In the process of stressing API in the presence of excipients, one should take into account the potential for physical change of the API and excipients (121) to occur in the formulation mixtures being evaluated. Depending on the stage of development and the depth of excipient compatibility studies, physical form assessment of either the API or excipients may be cursory (for early exploratory stage investigations) or more extensive and complete (for later development stage investigations). The following section focuses mainly on physical form assessment of the API, but the same physical form assessment regimens can be applied to understand the physical form changes of the excipients also.

Physical form changes such as crystallization of amorphous API, polymorphic conversions, conversions of API to hydrate or solvate forms, and conversion of crystalline forms to amorphous forms are some common phase transitions that may occur under the conditions of excipient compatibility and accelerated testing. Assessment of physical form changes should be directly linked to chemical stability data to obtain the best overall view of compatibility. If tracking decomposition kinetics in early formulation studies, knowledge of physical form integrity is essential before kinetic analysis can be applied. It is always advantageous to begin to track physical form integrity in the early excipient compatibility studies to avoid significant problems occurring in later development stages (122,123).

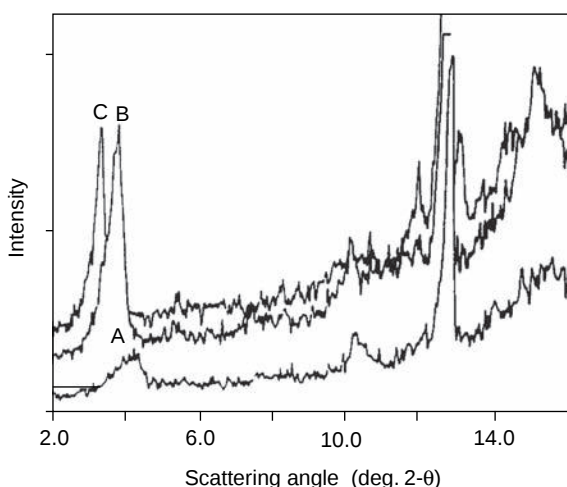


Figure 12 Physical changes occurring to SQ-33600 di-sodium salt during solid formulation processes as described using PXRD analysis. Sample A represents the initial wet-granulated amorphous material. Samples B and C represent samples following storage at 52% RH and 75% RH, respectively.

In the case studies examined in this section, a combination of several analytical methods is utilized to fully characterize physical form changes of API in excipient mixtures following preparation and accelerated stress testing. Some common solid-state methods used to study the physical forms of API in solid dosage formulation matrices include Raman spectroscopy (124), powder x-ray diffraction (PXRD) (125), reflectance FT-IR spectroscopy (126), thermal analysis (127), solid-state nuclear magnetic resonance (NMR) (128), and chemical imaging (13). It is recommended that these methods be used in combination with each other to describe physical changes of the API during excipient compatibility testing and accelerated stress conditions. The results of these techniques are often correlated with auxiliary analytical methods such as light microscopy/hot stage microscopy, scanning electron microscopy (SEM) and dissolution and solubility assessments.

Following complete characterization of the physical form stability of the API alone, Serajuddin et al. (12) traced the physical form changes of SQ-33600 disodium salt through the solid dosage formulation process. Using powder x-ray diffractions studies, it was revealed that the API turned amorphous upon initial wet granulation (Fig. 12, curve A). This finding led to a dry granulation process being utilized for initial processing of SQ-33600. Thermal-humidity challenges of prototype capsules and tablets of both wet and dry granulated materials were completed and powder X-ray analysis again detailed changes in the crystal form. The amorphous material was shown to convert to crystalline hydrate on further exposure to several high relative humidity stress conditions, such as 52% RH and 75% RH. Figure 12 shows the PXRD analysis of the prepared granules following humidity challenge (curves B and C). This example shows the necessity of tracking changes in the physical form of the API through initial formulation processes and following accelerated stability challenges. Supporting dissolution studies were effectively utilized to determine that the physical form change was expected to have limited impact on the performance of the proposed final dosage form.

More recently, Markovich et al. utilized a combination of solid-state IR and NMR methods to study the amorphous to crystalline API transition of SCH 48461 in solid dispersion capsule formulations (129). In this illustrative study, dissolution testing initially revealed inter and intra lot variations of capsules stored under accelerated stability conditions (25°C/60% RH, 30°C/60% RH, and 40°C/80% RH). Powder X-ray diffraction analysis could not explain the dissolution data being collected on lots stored at accelerated conditions and revealed no differences from

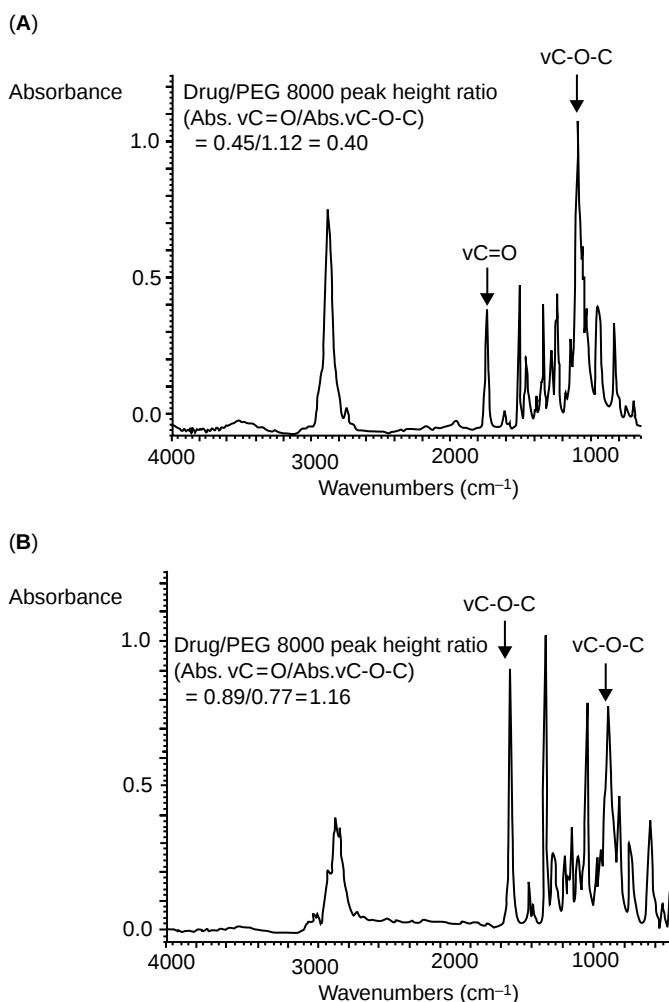


Figure 13 Comparison of the ATR-IR carbonyl and ether band intensities of initially prepared SCH 48461 capsule cores of **(A)** rapidly dissolving capsule lot (lot A) and **(B)** slower dissolving capsule lot (lot C).

original diffraction patterns. Two additional analytical techniques, attenuated total reflectance infrared (ATR-IR) spectroscopy and solid-state ^{13}C nuclear magnetic resonance (^{13}C NMR) spectroscopy, were employed to study the physical form in the actual solid dispersion formulations.

Because amorphous and crystalline solid-state forms contain nonequivalent spatial relationships at the molecular level, they often display differences in functional group vibrational modes that can be measured by infra-red (IR) spectroscopy. Total attenuated reflectance IR spectroscopy is utilized because it is nondestructive and can be used to directly measure actual tablet and capsule samples. Similarly, solid-state NMR spectroscopy is another nondestructive direct analytical method that can detect and measure differences in nuclear resonance frequencies and relaxations, such as those displayed by amorphous and crystalline material. Cross-polarization magic angle spinning (CP-MAS) variations enhance the power of solid-state NMR and involve (i) strong proton decoupling, which eliminates coupling to neighboring proton nuclei, thereby simplifying the acquired spectra, (ii) utilizing rapid magic angle spinning to remove random orientation effects, and (iii) utilizing nuclear cross-polarization techniques to provide stronger signal-to-noise ratios and faster acquisitions times.

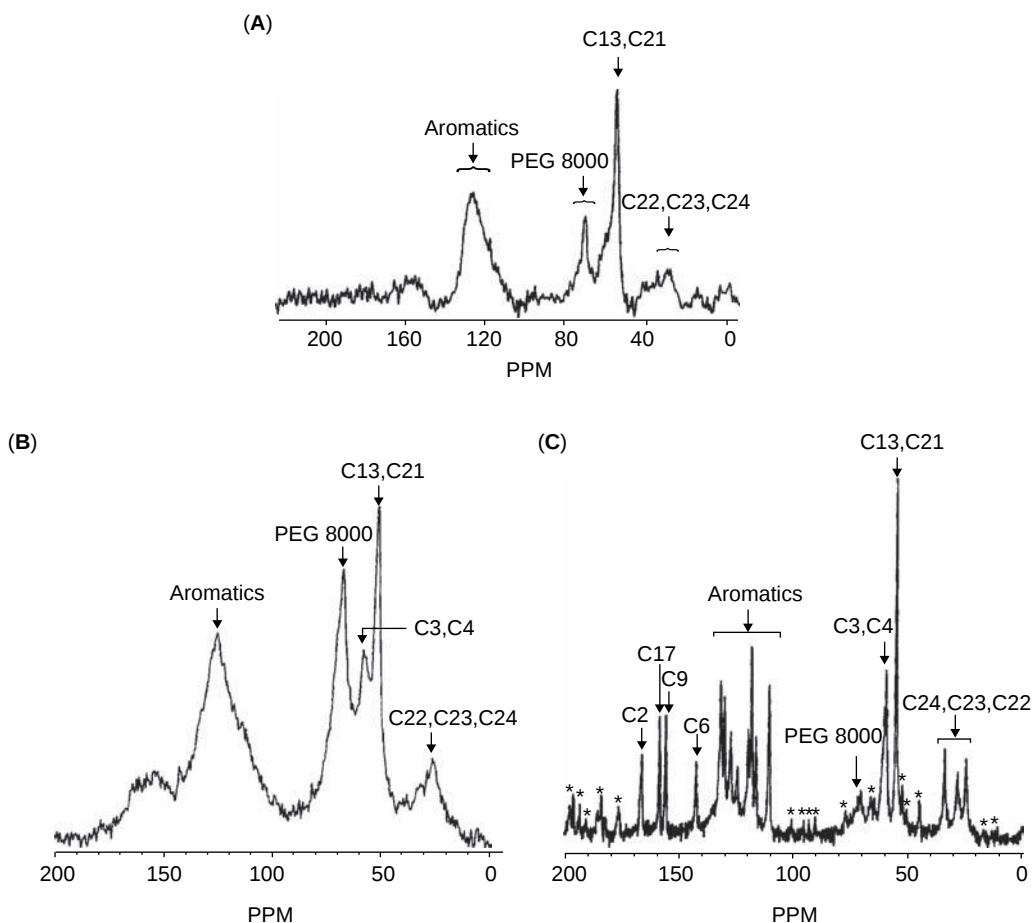


Figure 14 Solid-state CP-MAS ^{13}C NMR spectra of intact capsule cores of SCH 48461 formulations (A) after storage for 6 months at room temperature, (B) after storage at $30^\circ\text{C}/60\%\text{RH}$ for 18 months, and (C) after storage for 24 months at room temperature.

ATR-IR analysis of the relative intensities of the strong carbonyl band of SCH 48461 (1740cm^{-1}) and the intense ether band of the polyethylene glycol 8000 excipient (1100cm^{-1}) were utilized to track differences in lots of formulated capsule cores [Fig. 13(A) and 13(B)]. The ratio of the intensity of these vibrations revealed large differences in two formulated lots of material, thereby effectively explaining the dissolution results previously obtained.

Solid state CPMAS ^{13}C -NMR analysis of the samples was used to confirm the data generated using the ATR-IR technique. Solid-state NMR spectra of SCH 48461 capsule cores following storage for 6 months at room temperature and after 18 month storage at $30^\circ\text{C}/60\%\text{RH}$ are shown in Figure 14 (spectra A and B). These spectra show typically broad signals associated with amorphous material. The obvious change in line width of the sample stored for 24 months at room temperature immediately suggests that a physical form change may have occurred, as narrow linewidths are more closely associated with crystalline materials (Fig. 14, spectrum C). In a series of further cross-polarization and interrupted-decoupling experiments, the presence of crystalline material in several lots of SCH-48461-PEG 8000 solid dispersion formulation preparations was confirmed.

The combination of solid-state ATR-IR and solid-state NMR data supported the conclusion that the presence of crystalline material was responsible for changes in the dissolution profiles of the different lots. The results appear consistent with historical examples of changes in API physical form of solid, high molecular weight, polyethylene glycol dispersion formulations of amorphous indomethacin and griseofulvin (130–132).

In the examples presented above, many techniques were utilized to obtain the fullest picture of the changes and interactions of the API in the formulation mixtures. Very often, these changes and interactions are linked to changes in chemical stability or performance of the dosage form such that some initial monitoring of these physical changes and interactions should be considered and incorporated in excipient compatibility testing when applicable.

KINETICS AND DECAY EXTRAPOLATION BASED ON EXCIPIENT COMPATIBILITY DATA

Kinetic information on the chemical changes of excipient compatibility samples is a direct outcome of most formulation compatibility studies. Because accelerated conditions of thermal and thermal humidity stress are employed, degradation will often occur at the selected conditions and this degradation can be monitored with time. Kinetic evaluation of the data can address the behavior and extent of decay such that degradation data can effectively be utilized to determine levels and conditions of compatibility (133). It is not the aim of this section to recommend a complete outline of a stability program, rather it is to describe simple concepts, and exercises that will help the excipient compatibility formulator utilize and apply their data most effectively. Even small studies having a limited amount of samples for analysis can yield useful and informative trends that can be valuable when designing a complete stability program.

For any kinetic analysis, the more distinct data points acquired the better characterization of the decay. This factor must be weighed against the resources available for the study, but the maximum number of data points should be collected whenever possible. It is recommended that multiple samples be analyzed per data point to rule out spurious results. An assessment of uniformity of prepared samples should be strongly considered. It is recommended that over the timeframes of the study, the number of samples and data points be rationally prescribed (88), such that higher temperature and humidity conditions include analysis of more early data points. Some reports (76) suggest that a temperature of $\sim 70^{\circ}\text{C}$ be the upper limit to employ for compatibility studies, as higher temperatures may not provide realistic and relevant degradation data for simple kinetic analyses. Realistic and relevant degradation data may not be gleaned from compatibility samples if the physical form and integrity of the excipient is lost at these elevated temperatures. Therefore, for some polymeric and specialty excipients, a maximum stress temperature of only 50°C , in addition with lowered humidity levels ($<75\%\text{RH}$), is typically recommended. Correspondingly, rigorous kinetic consideration of largely degraded samples ($>50\%$ decay) should be avoided, as secondary degradation reactions and involvement of decay products in further decay reactions can convolute analysis. For highest quality kinetic data, all samples should be analyzed immediately after sampling using appropriately sensitive analytical methodology and not stored for long periods of time.

Often, different decay kinetics are observed in bulk API stability and excipient compatibility studies because the system changes from a homogeneous solid mixture to a heterogeneous, multicomponent mixture involving gas, liquid, and solid phases (134). For initial consideration, total loss of API, loss of API via a specific major degradation mechanism or growth of a major, significant degradation product with time can be investigated kinetically. For emphasis, it should be restated that prior to kinetic analysis, confirmation of API physical form integrity in the excipient samples is recommended. Modeling of chemical API decay may be severely convoluted by the kinetics of physical form transformations.

Kinetic data treatment may begin with a plot of potency or growth of a major degradation product with respect to time, for a given set of temperature and humidity conditions. If the data is best fit by a simple linear plot of these separate parameters with time, the degradation in the

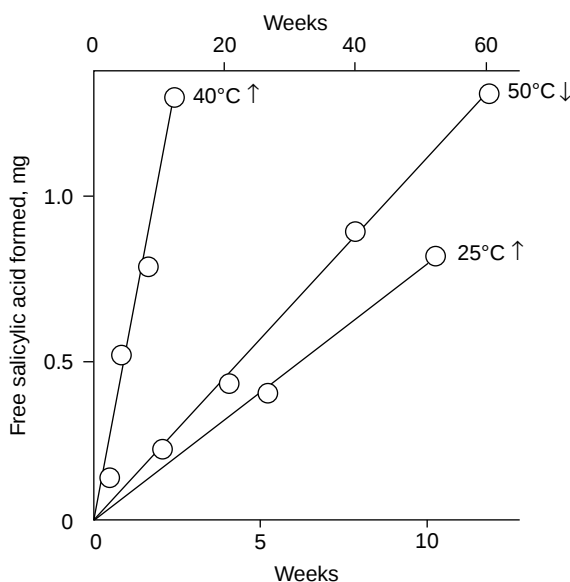


Figure 15 The kinetics of formation of salicylic acid from the decomposition of aspirin in the solid state at varying temperature (Note: arrows direct the use of either upper or lower time axis at each specified storage temperature).

excipient mixture is likely following zero-order kinetics. The example detailed in Figure 15 illustrates the kinetics of formation of free salicylic acid from the decomposition of aspirin in the solid state. Analysis reveals that at three separate temperatures, the formation of the degradation product is linear with respect to time, with the slope of the line being the temperature dependant rate constant of the reaction. Zero-order reactions effectively model degradation occurring in saturated adsorbed moisture layers of the drug or excipient. It should be noted that degradation profiles can often appear zero order at low degradation levels (up to ~10% degradation formation) and the kinetic order may only clearly distinguish itself at degradation product formation of *circa* 30–50% (135). For systems where the degradation kinetics are completely unknown, i.e., during initial compatibility studies, both shorter and longer time samples are helpful in characterizing the true nature of the kinetics of degradation.

A common decay pattern of water soluble drug substances in the presence of excipients is exemplified by the degradation of thiamine hydrochloride in microcrystalline cellulose-magnesium stearate tablets as described by Carstensen (136) and detailed in Figure 16. Here, the stability profile shows initial rapid decay followed by a leveling to an “equilibrium” degradation level $[A_{\infty}]$, which was determined to be dependent on the amount of water present in the matrix. For the early degradation kinetics (up to day 7), a first order decay character was observed and a plot of the log of the remaining thiamine hydrochloride per tablet at a specific point $[A]$ in relation to the observed equilibrium degradation level $[A_{\infty}]$ with respect to time (in days) yielded a linear slope (Fig. 17).

Rapid first-order degradation decay followed by a subsequent slowing or halt of degradation is a common observation in compatibility studies and there are several scenarios for which this behavior may be observed. In the case of reactive impurities present in the excipients, the level at which degradation slows for all samples of the same composition will be identical, while the rate at which they achieve that level will vary with temperature. Rapid first-order decay that slows can often be attributed to the presence of a small but significant amount of reactive material present in the API. This phenomenon can often be attributed to API that is in a higher energy crystalline state (different polymorph, water hydrate, or solvate), crystalline material that is unstable because it is in the vicinity of crystal defect areas, or API that is present

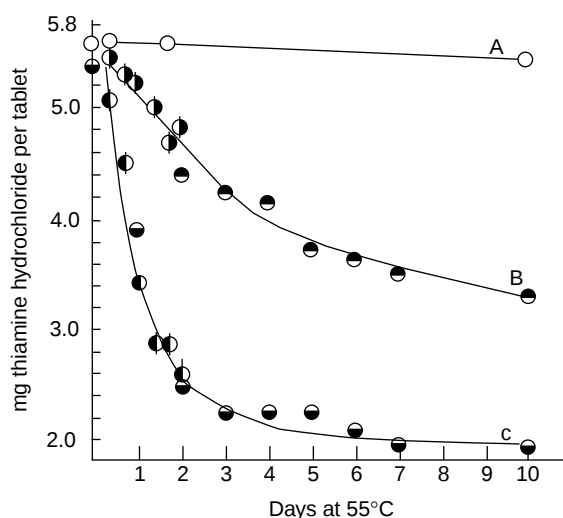


Figure 16 Stability of thiamine hydrochloride in a cellulose-magnesium stearate tablet containing various amounts of moisture (curve A = no moisture added, curve B = 1% moisture added, and curve C = 3% moisture added) following storage at 55°C.

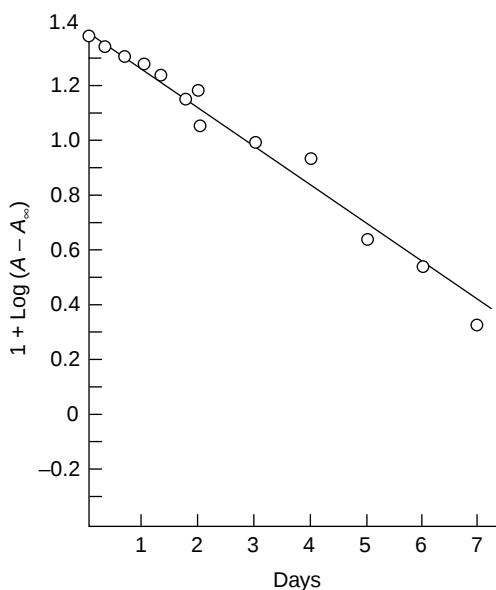


Figure 17 Plot of the logarithm of thiamine hydrochloride content after the subtraction of equilibrium degradation product content ($A - A_{\infty}$) as a function of time at 55°C with addition of 4% moisture.

in a higher energy (amorphous) or even completely dissolved state. As described in several examples previously, it may be advantageous to design processes into excipient compatibility studies that promote the generation of reactive defects and/or amorphous material in order to observe effects. Typically, rapid decay is noted until the amount of solublized or amorphous material or impurity or defects are depleted, then the decay slows. This phenomenon imparts heterogeneity to the reaction kinetics. Finally, direct solid-solid chemical reaction decay routes

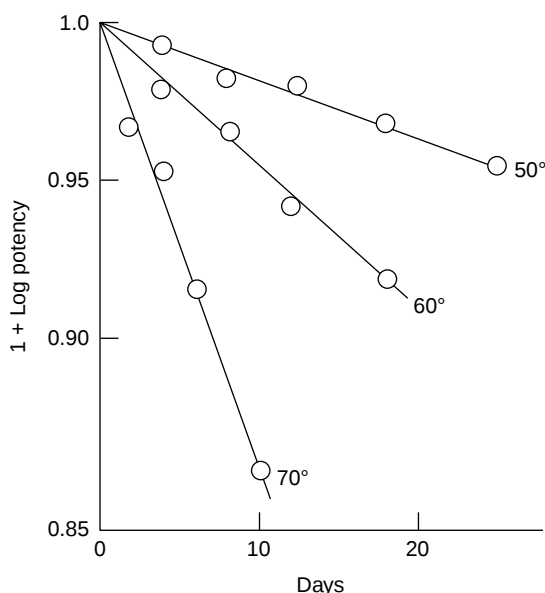


Figure 18 Pseudo-first-order plot of thermal degradation of ascorbic acid (vitamin C) in a solid dosage form at various temperatures (50–70°C).

that exhibit first-order kinetics can illicit observation of decay that is eventually limited by the growth of the impurities at the surfaces of the reacting solids. In such specific cases, the decay level may closely relate to the surface area of the API and/or reactive excipient.

If first or pseudo-first-order decay is observed throughout the length of the compatibility study, temperature dependent rate constants can be easily determined (or at least estimated). If a plot of these rate constants versus reciprocal temperature ($1/T$) produces a linear correlation, the system is adhering to the well-studied Arrhenius kinetic model and easy prediction of the rate of decay at any temperature can be made. For example, Carstensen's manipulation of decay data, originally described by Tardif (137), demonstrates the pseudo-first-order decay behavior of the decomposition of ascorbic acid in solid dosage forms at temperatures of 50°C, 60°C, and 70°C under sealed conditions (138) (Fig. 18). Analysis of the data confirmed that the system adhered closely to Arrhenius behavior as the plot of the temperature-dependant rate constants with respect to reciprocal temperature ($1/T$) showed adequate linearity (Fig. 19).

Generally, Arrhenius behavior is demonstrated in systems where the relative humidity was kept constant and only the temperature value varied. Performing stability experiments at several constant humidities, while varying temperature in each case, can significantly increase the number of compatibility samples that are needed to demonstrate Arrhenius behavior, just so that a standard Arrhenius based prediction can be made. This resource intensive scenario may not be the best "fit for purpose" evaluation during early investigations into excipient compatibility studies.

More recently, the development of a humidity corrected Arrhenius model has been reported (135,139). In this case, an additional term (B) has been added to the Arrhenius equation and represents the fact that the humidity effects can largely be treated independent of the temperature effects (133). This correlation effectively demonstrated that there is no cross term of temperature and humidity interactions needed to describe chemical degradation of drug substance and drug product decay kinetics in the solid state. From the study of a diverse selection of drug substance and drug product systems, activation energies (E_a) ranged from

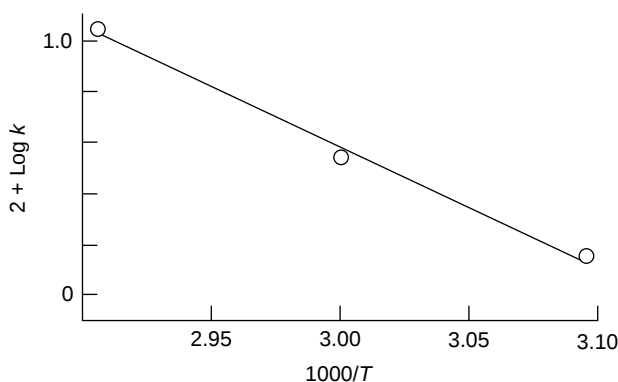


Figure 19 Arrhenius plots of the temperature-dependant rate constants derived from Figure 18.

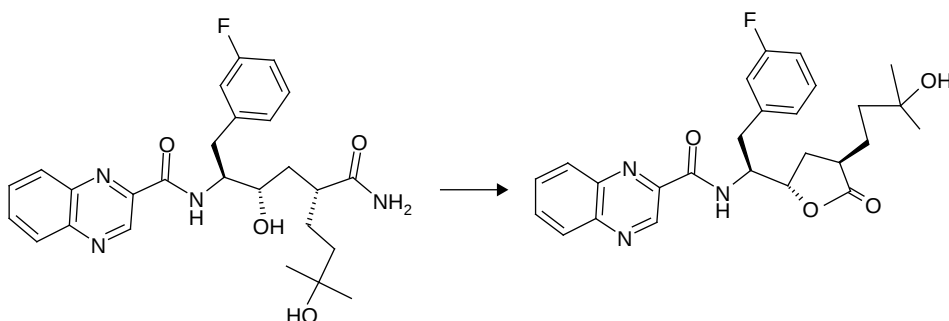


Figure 20 Intramolecular cyclization degradation reaction of CP-481,715 in oral dosage forms modeled in the *iso-conversion* paradigm.

16 to 27.7 kcal/mol, with the median value being 22 kcal/mol and the B values ranged from 0.01 to 0.08, with the median value being 0.04 (140).

For example, the degradative cyclization of CP-481,715 to the lactone (Fig. 20) was modeled using the isoconversion paradigm in several different immediate release and controlled release dosage forms. The degradation of CP-481,715 in an immediate release dosage form demonstrated a linear response of degradation ($\ln k$) versus percent relative humidity (5–75%RH) at 40°C (B value = 0.069). The shelf-life estimate (90% confidence) for 0.2% degradation of crystalline CP-481,715 formulated in immediate release dosage forms at 30°C/60%RH was extrapolated to be 3.7+/-0.3 days, using the humidity corrected Arrhenius equation. The actual, experimental time for 0.2% degradation of crystalline CP-481,715 in a solid dosage formulation was 3.2+/-0.1 days, indicating the validity of this prediction approach.

This correlation is currently being applied to designing compatibility stability studies under isoconversion paradigms, which lead to the use of nonstandard (>70°C) conditions, specifically dictated by the decay characteristics of the given system. This approach results in shelf life expiry determinations in just two weeks, compared to months using a conventional stability protocol.

Review of compatibility data in this manner is important and useful in many cases. The adherence of decay to Arrhenius and/or humidity corrected Arrhenius kinetics provides the formulator with a powerful tool for prediction and understanding of degradation. Short-term

accelerated stress conditions can efficiently be used to extrapolate the amount of decay expected at realistic long-term storage conditions. The Arrhenius decay model remains the basis for the International Committee on Harmonization (ICH) quality guidelines describing stability testing for new drug substances and products followed by the Food and Drug Administration (FDA) and the entire pharmaceutical industry (86). Efforts are currently underway to speed the utilization of the resource-sparing isoconversion paradigms and the humidity corrected Arrhenius kinetic predictions in designing and interpreting all excipient compatibility screening and setting initial shelf life designation in regulatory documents, such as investigational new drug (IND) applications.

CONCLUSION

Any excipient compatibility testing can become a complicated and extensive series of experiments. It has been questioned by Monkhouse (141) if these studies, in any form, are predictive enough to be worthy of the large experimental expense. To address this, several groups are working toward the automation of excipient compatibility testing and high throughput approaches (142). Effective utilization of these more modern approaches will lead to several advantages include the following:

- The efficient analysis of the most relevant distinct data points, so that kinetic analysis will be fully empowered
- The ability to evaluate and test all relevant experimental parameters to provide the best overview of compatibility
- The ability to evaluate processing parameters at each stage, leading to the identification of the most optimal processes
- Instilling a quality by design approach to compatibility testing at each development milestone and consider only “fit for purpose” experiments that coincide with the appropriate stage of development

Because a significant degree of resources are involved in generating data, it is highly recommended that compatibility data be compiled and referenced in complete, searchable databases. Databases will prove useful in later stages of development as a valuable reference to formulators looking to switch or exchange excipients in commercial formulations and for early stage formulators who may be examining compatibility of similar classes of compounds and molecule scaffolds. While this concept of rigorous mining of excipient compatibility data is not novel, there are only limited references to these types of database systems in the literature (143).

Overall, the concepts presented in this summary suggest that (i) an initial understanding of the properties of the API and excipients should be utilized to design excipient compatibility studies, (ii) the understanding of the composition of the API-excipient mixtures (water, impurities, metals, etc.) should be considered when performing compatibility testing, and (iii) the physical form of the API and excipients in the solid state need to be considered when interpreting compatibility data.

REFERENCES

1. Whittet T. Decomposition of medicaments due to excipients and containers and its prevention. *Pharm Acta Helv* 1959; 34: 489–520.
2. Stahl VPH. Die Charakterisierung und Aufbereitung des Wirkstoffs bei der Entwicklung fester Arzneiforme. *Pharm Ind* 1989; 51: 425–39.
3. Dudinkski J, Lachman L, Shami E, Tingstad J. Preformulation studies: I. Molindone hydrochloride. *J Pharm Sci* 1973; 62: 622–4.
4. Alsante KM, Martin L, Baertschi SW. Stress testing benchmarking study. *Pharm Tech* 2003; 27: 60–72.
5. Baertschi SW, Alsante K. Stress testing: the chemistry of drug degradation. In: Baertschi SW, ed. *Pharmaceutical Stress Testing: Predicting Drug Degradation*, chap. 3. 1st ed. Boca Raton, FL: Taylor & Francis, 2005: 482.

6. Jorgensen WL, Laired ER, Gushurst AJ, et al. CAMEO: a program for the logical prediction of the products of organic reaction. *Pure Appl Chem* 1990; 62: 1921–32.
7. Lhasa Limited. Zeneth Software (<https://www.lhasalimited.org/zeneth/>), 2010.
8. Alsante KM, Ando A, Brown R, et al. The role of degradant profiling in active pharmaceutical ingredients and drug products. *Adv Drug Delivery Rev* 2007; 59: 29–37.
9. Paul IC, Curtin DY. Thermally induced organic reactions in the solid state. *Acc Chem Res* 1973; 6: 217–25.
10. Byrn SR, Xu W, Newman A. Chemical reactivity in solid-state pharmaceuticals: formulation implications. *Adv Drug Delivery Rev* 2001; 48: 115–36.
11. Guerrieri PP, Smith DT, Taylor LS. Phase behavior of ranitidine HCl in the presence of degradants and atmospheric moisture—impact on chemical stability. *Langmuir* 2008; 24: 3850–6.
12. Serajuddin ATM, Morris KR, Newman AW, et al. Characterization of humidity-dependent changes in crystal properties of a New HMG–CoA reductase inhibitor in support of its dosage form development. *Int J Pharm* 1994; 108: 195–206.
13. Bugay DE. Characterization of the solid-state: spectroscopic techniques. *Adv Drug Delivery Rev* 2001; 48: 43–65.
14. United States Food and Drug Administration. Q1B: Stability Testing: Photostability Testing of New Drug Substances and Products. Federal Register 1996: 1–8.
15. Filho ROC, Franco PIBM, Conceicao EC, Leles MIG. Stability studies on nifedipine tablets using thermogravimetry and DSC. *J Therm Anal Calorim* 2009; 97: 343–7.
16. York P. Solid-state properties of powders in the formulation and processing of solid dosage forms. *Int J Pharm* 1983; 17: 1–28.
17. Teraoka R, Otsuka M, Matsuda Y. Evaluation of photostability of solid-state dimethyl 1,4-dihydro-2,6-dimethyl-4-(2-nitro-phenyl)-3,5-pyridinecarboxylate by Fourier-transformed reflection-absorption infrared spectroscopy. *Int J Pharm* 1999; 184: 35–43.
18. Harris KDM. New opportunities for structure determination of molecular materials directly from powder diffraction data. *Crys Growth Des* 2003; 3: 887–95.
19. David WIF, Shankland K. Structure determination from powder diffraction data. *Acta Cryst* 2008; A64: 52–64.
20. Wray PE. The physics of tablet compaction revisited. *Drug Dev Ind Pharm* 1992; 18: 627–58.
21. Lefebvre C, Guyot-Hermann AM. Polymorphic transitions of carbamazepine during grinding and compression. *Drug Dev Ind Pharm* 1986; 12: 1913–27.
22. Pikal MJ, Lukes AL, Lang JE. Thermal decomposition of amorphous beta-lactam antibacterials. *J Pharm Sci* 1977; 66: 1312–16.
23. Waltersson J, Lundgren P. The effect of mechanical comminution on drug stability. *Acta Pharm Suec* 1985; 22: 291–300.
24. Morris KR, Griesser UJ, Eckhardt CJ, Stowell JG. Theoretical approaches to physical transformations of active pharmaceutical ingredients during manufacturing processes. *Adv Drug Delivery Rev* 2001; 48: 91–114.
25. Chiou KL, Kyle LE. Differential thermal, solubility and aging studies on various sources of digoxin and digitoxin powder: biopharmaceutical implications. *J Pharm Sci* 1979; 68: 1224–9.
26. Konty MJ. Distribution of water in solid pharmaceutical systems. *Drug Dev Ind Pharm* 1988; 14: 1991–2027.
27. Pikal M, Dellerman K. Stability testing of pharmaceuticals by high-sensitivity isothermal calorimetry at 25°C: cephalosporins in the solid and aqueous solution states. *Int J Pharm* 1989; 50: 233–52.
28. Koenigbauer MJ, Brooks SH, Rullo G, Couch RA. Solid-state stability testing of drugs by isothermal calorimetry. *Pharm Res* 1992; 9: 939–44.
29. Buckton G, Gaisford S. The use of microcalorimetry in stress testing. In: Baertschi SW, ed. *Pharmaceutical Stress Testing: Predicting Drug Degradation*, chap. 11. 1st edn. Boca Raton, FL: Taylor & Francis Group, 2005: 482.
30. Brittain HG. X-ray diffraction: III. Pharmaceutical application of x-ray powder diffraction. *Pharm Tech North Am* 2001; 25: 142–50.
31. Suryanarayanan R, Herman CS. X-ray powder diffractometry. *Drug Pharm Sci* 1995; 70: 187–221.
32. Florence AT, Salole EG. Changes in crystallinity and solubility on comminution of digoxin and observations on spironolactone and oestradiol. *J Pharm Pharm* 1976; 28: 637–42.
33. Byrn SR, Lin C. The effect of crystal packing and defects on desolvation of hydrate crystals of caffeine and L-(–)-1,4-cyclohexadien-1-alanine. *J Am Chem Soc* 1976; 98: 4004–5.

34. Clay RJ, Knevel AM, Byrn SR. The desolvation and oxidation of crystals of dialuric acid monohydrate. *J Pharm Sci* 1982; 71: 1289–91.
35. Halebilian J, McCrone W. Pharmaceutical application of polymorphism. *J Pharm Sci* 1969; 58: 911–29.
36. Aruga M, Awazu A, Hanano M. Kinetic studies on decomposition of glutathione: I. Decomposition in the solid state. *Chem Pharm Bull* 1978; 26: 2081–91.
37. Zhang GGZ, Zhou D. Crystalline and amorphous solids. In: Qiu Y, Chen Y, Zhang GGZ, Liu L, Porter W, eds. *Developing Solid Oral Dosage Forms—Pharmaceutical Theory and Practice*, chap. 2. 1st edn. Burlington, MA: Academic; 2009: 978.
38. Kibbe AH, ed. *Handbook of Pharmaceutical Excipients*. 3rd ed. Washington, DC: American Pharmaceutical Association, 2000.
39. Bugay DE, Findlay WP, eds. *Pharmaceutical Excipients: Characterization by IR, Raman and NMR Spectroscopy*. New York, NY: Marcel Dekker, 1999.
40. Hancock BC, Shamblin SL. Water vapour sorption by pharmaceutical sugars. *Pharm Sci Tech Today* 1998; 1: 345–51.
41. Govindarajam R, Zinchuk A, Hancock B, Shalaev E, Suryanarayanan R. Ionization States in the microenvironment of solid dosage forms: effect of formulation variables and processing. *Pharm Res* 2006; 23: 2454–68.
42. Lundgren PA. Methods for the evaluation of solid state stability and compatibility between drug and excipient. *Acta Pharm Suecica* 1985; 22: 305–14.
43. Patel NK, Patel IJ, Cutie AJ et al. The effect of selected direct compression excipients on the stability of aspirin as a model hydrolyzable drug. *Drug Dev Ind Pharm* 1988; 14: 77–98.
44. Chen J-G, Markovitz DA, Yang AY, et al. Degradation of a fluoropyridinyl drug in capsule formulation: degradant identification, proposed degradation mechanism and formulation optimization. *Pharm Dev Tech* 2000; 5: 561–70.
45. Castello RA, Mattocks AM. Discoloration of tablets containing amines and lactose. *J Pharm Sci* 1962; 51: 106–8.
46. Wirth DD, Baertschi SW, Johnson RA, et al. Maillard reaction of lactose and fluoxetine hydrochloride, a secondary amine. *J Pharm Sci* 1998; 87: 31–9.
47. Harmon PA, Yin W, Bowen WE, Tyrrell RJ, Reed RA. LC-MS and ¹H NMR characterization of trace level condensation products formed between lactose and the amine containing diuretic hydrochloride. *J Pharm Sci* 2000; 7: 920–9.
48. Brewster ME, Loftson T. The use of chemically modified cyclodextrins in the development of formulations for chemical delivery systems. *Pharmazie* 2002; 57: 94–101.
49. Alvarez C, Calero J, Menendez JC, Torrado S, Torrado JJ. Effects of hydroxypropyl-beta-cyclodextrin on the chemical stability and the aqueous solubility of thalidomide enantiomers. *Pharmazie* 2008; 63: 511–13.
50. Higuchi T, Lachman L. Inhibition of hydrolysis of esters in solution by formation of complexes I. *J Am Pharm Assoc* 1955; 44: 521–6.
51. Bhattacharya S. Influence of intact and hydrolyzable protein in solid state. *J Inst Chem Calcutta* 1969; 41: 147–53.
52. Thoma K, Klimek R. Photostabilization of drugs in dosage forms without protection from packaging material. *Int J Pharm* 1991; 67: 169–75.
53. Crowley PJ. Excipients as stabilizers. *Pharm Sci Tech Today* 1999; 2: 237–43.
54. Bayomi B, Abanumay KA, Al-Angary AA. Effect of inclusion complexation with cyclodextrins on photostability of nifedipine in solid state. *Int J Pharm* 2002; 243: 107–17.
55. Thoma K, Spilgies H. Photostabilization of solid and semisolid dosage forms. In: Piechocki JT, Thoma K, eds. *Pharmaceutical Photostability and Stabilization Technology*. New York, NY: Informa Healthcare, 2007: 445.
56. Moribe K, Sekiya N, Fujito T, et al. Stabilization mechanism of limaprost in solid dosage form. *Int J Pharm* 2007; 338: 1–6.
57. Nieuwmeyer F, Maarchalk K V, Vromans H. Lactose contaminant as steroid degradation enhancer. *Pharm Res* 2008; 25: 2666–73.
58. Jansen PJ, Oren PL, Kemp CA, Maple SR, Baertschi SW. Characterization of impurities found by interaction of duloxetine HCL with enteric polymers hydroxypropyl methyl cellulose acetate succinate and hydroxypropyl methylcellulose phthalate. *J Pharm Sci* 1998; 87: 81–5.
59. Wasylaschuk W, Harmon PA, Wagner G, et al. Evaluation of hydroperoxides in common pharmaceutical excipients. *J Pharm Sci* 2007; 96: 106–16.

60. Hartauer KJ, Arbuthnot GN, Baertschi SW, et al. Influence of peroxide impurities in povidone and crospovidone on the stability of raloxifene hydrochloride in tablets: identification and control of an oxidative degradation product. *Pharm Dev Tech* 2000; 5: 303–10.
61. Yue H, Bu X, Huang M-H, Young J, Raglione T. Quantitative determination of trace levels of hydrogen peroxide in crospovidone and a pharmaceutical product using high performance liquid chromatography with coulometric detection. *Int J Pharm* 2009; 375: 33–40.
62. Harmon PA, Wuelfing WP, Harman AB et al. Role of organic hydro-peroxides on process robustness and finished product quality. In: American Association of Pharmaceutical Scientists 2004 Annual Meeting (AAPS) November 07–11, 2004; Baltimore, Maryland USA.
63. Li Z, Jacobus LK, Wuelfing WP et al. Detection and quantification of low-molecular-weight aldehydes in pharmaceutical excipients by headspace gas chromatography. *J Chromatogr A* 2006; 1104: 1–10.
64. Li Z, Kozlowski BM, Chang EP. Analysis of aldehydes in excipients used in liquid/semi-solid formulations by gas chromatography-negative chemical ionization mass spectrometry. *J Chromatogr A* 2007; 1160: 299–305.
65. Brittain HG, Bogdanowich SJ, Bugay DE et al. Physical characterization of pharmaceutical solids. *Pharm Res* 1991; 8: 963–73.
66. Jones TM. The physico technical properties of starting materials used in tablet formulation. *Int J Pharm* 1981; 2: 17–24.
67. Brittain HG, Fiese EF. Effect of pharmaceutical processing on drug polymorphs and solvates. In: Brittain HG, ed. *Polymorphism in Pharmaceutical Solids*. New York: Marcel Dekker, 1999: 331–62.
68. Yu LX, Furness MS, Raw A, et al. Scientific considerations of pharmaceutical solid polymorphism in abbreviated new drug applications. *Pharm Res* 2003; 20: 531–6.
69. Eyjolfsson R. Enalapril maleate polymorphs: instability of form II in a tablet formulation. *Pharmazie* 2002; 57: 347–8.
70. Begat P, Young PM, Edge S, Kaerger JS, Price R. The effect of mechanical processing on surface stability of pharmaceutical powders: visualization by atomic force microscopy. *J Pharm Sci* 2003; 92: 611–20.
71. Monkhouse DC, Maderich A. Excipient compatibility possibilities and limitations in stability prediction. In: *Stability Testing in the EC, Japan and the USA*. Vol. 32 Paperback APV, 1993: 67–74.
72. Leuenberger H, Becher W. A factorial design for compatibility studies in preformulation work. *Pharm Acta Helv* 1975; 50: 88–91.
73. El-Banna HM, Ismail AA, Gadalla MAF. Factorial design of experiment for stability studies in the development of a tablet formulation. *Pharmazie* 1984; 39: 163–5.
74. Durig T, Fassihi AR. Identification of stabilizing and destabilizing effects of excipient-drug interactions in solid dosage form design. *Int J Pharm* 1993; 97: 161–70.
75. Baertschi SW, Jansen P. Stress testing: a predictive tool. In: Baertschi SW, ed. *Pharmaceutical Stress Testing: Predicting Drug Degradation*, chap 2. 1st edn. Boca Raton, FL: Taylor & Francis Group, 2005: 482.
76. Sims J, Carreira J, Carrier D, et al. A new approach to accelerated drug–excipient compatibility testing. *Pharm Dev Tech* 2003; 8: 119–26.
77. Badway S, Williams R, Gilbert D. Chemical stability of an ester prodrug of a glycoprotein IIb/IIIa receptor antagonist in solid dosage forms. *J Pharm Sci* 1999; 88: 428–33.
78. Guo Y, Byrn S, Zografi G. Physical characteristics and chemical degradation of amorphous quinapril hydrochloride. *J Pharm Sci* 2000; 89: 128–43.
79. Leung SS, Grant DW. Solid state stability studies of model dipeptides: aspartame and aspartylphenylalanine. *J Pharm Sci* 1997; 86: 64–71.
80. Gu L, Strickley RG. Diketopiperazine formation, hydrolysis, and epimerization of the new dipeptide angiotensin-converting enzyme inhibitor RS-10085. *Pharm Res* 1987; 4: 392–7.
81. Polizzi MA, Singhal D, Colvin J. Mechanoradical-induced degradation in a pharmaceutical blend. *Pharm Dev Technol* 2008; 13: 547–462.
82. Masayuki K, Yamauchi Y, Kondo S-i. Mechanolysis of glucose-based polysaccharides as studied by electron spin resonance. *J Phys Chem B* 1999; 109: 8051–9.
83. Boccardi G. Oxidative susceptibility testing. In: Baertschi SW, ed. *Pharmaceutical Stress Testing: Predicting Drug Degradation*, chap. 7. 1st edn. Boca Raton, FL: Taylor & Francis Group, 2005: 482.
84. Ahlneck C, Lundgren P. Methods for the evaluation of solid state stability and compatibility between drug and excipient. *Acta Pharm Suec* 1986; 22: 305–14.

85. Serajuddin A, Thakur A, Ghoshal R, et al. Selection of solid dosage form composition through drug-exciipient compatibility testing. *J Pharm Sci* 1999; 88: 696–704.
86. United States Food and Drug Administration. Q1A(R2) Stability Testing of New Drug Substances and Product. Federal Register 2003: 65717–18.
87. Eyjolfsson R. Diclofenac sodium: oxidative degradation in solution and solid state. *Drug Dev Ind Pharm* 2000; 26: 451–3.
88. Carstensen JT, ed. Stability of solid dosage forms, in progress in the quality control of medicines. Amsterdam: Elsevier Biomedical Press. 1981.
89. Munday R, Munday CM, Winterbourn CC. Inhibition of copper-catalyzed cysteine oxidation by nanomolar concentrations of iron salts. *Free Rad Biol Med* 2004; 36: 757–64.
90. Waterman K, Adami R, Alsante K, et al. Stabilization of pharmaceuticals to oxidative degradation. *Pharm Dev Tech* 2002; 7: 1–32.
91. Yoshioka S, Ogata H, Shibasaki T, Ejima A. Stability of sulpyrine: V. Oxidation with molecular oxygen in the solid state. *Chem Pharm Bull* 1979; 27: 2363–71.
92. Mitsubishi Gas Chemical Company of Japan. Ageless oxygen absorber preserving Product, Purity, Integrity and Freshness. Tokyo, 1994.
93. Mahajan R, Templeton A, Harman A, Reed RA, Chern R. The effect of inert atmospheric packaging on oxidative degradation in formulated granules. *Pharm Res* 2005; 22: 128–35.
94. Waterman K, Adami R, Alsante K, et al. Hydrolysis in pharmaceutical formulations. *Pharm Dev Tech* 2002; 7: 113–46.
95. Carstensen JT, Johnson JB, Valentine W, Vance JJ. Extrapolation of appearance of tablets and powders from accelerated storage tests. *J Pharm Sci* 1964; 53: 1050–4.
96. Wirth M. Instrumental color measurement: a method for judging the appearance of tablets. *J Pharm Sci* 1991; 80: 1177–9.
97. Stark G, Fawcett JP, Tucker IG, Weatherall IL. Instrumental evaluation of color of solid dosage forms during stability testing. *Int J Pharm* 1996; 143: 93–100.
98. Hong D, Shah M. Development and validation of HPLC stability-indicating assay. In: Carstensen JT, ed. *Drugs and the Pharmaceutical Sciences*. New York: Marcel Dekker, 2000: 329–84.
99. Olsen B, Baertschi S, Riggin R. Multidimensional evaluation of impurity profiles for generic cephalixin and cefaclor antibiotics. *J Chromatogr* 1993; 648: 165–73.
100. Nussbaum M, Jansen P, Baertschi S. Role of “Mass balance” in pharmaceutical stress testing. In: Baertschi SW, ed. *Pharmaceutical Stress Testing: Predicting Drug Degradation*, chap. 6. 1st ed. Boca Raton, FL: Taylor & Francis Group, 2005: 482.
101. Alsante KM, Hatajik TD, Santafianos D, Sharp T, Lohr L. Solving impurity/degradation problems: case studies. In: Ahuja S, Alsante K, eds. *Handbook of Isolation and Characterization of Impurities in Pharmaceuticals*, chap. 14. San Diego, CA: Academic, 2003: 414.
102. Kuiken J, Mitchell T, Mann T, Burke M. SPE Method improves drug analysis. *Drug Disc Dev* 2000; 75–8.
103. Moreno P, Salvado V. Determination of eight water- and fat-soluble vitamins in multi-vitamin pharmaceutical formulations by high-performance liquid chromatography. *J Chromatography A* 2000; 870: 207–15.
104. Landis MS. The use of mixed-mode ion-exchange solid phase extraction to characterize pharmaceutical drug degradation. *J Pharm Biomed Analysis* 2007; 44: 1029–39.
105. Dorman D, Lorenz L, Ocolowitz J, et al. Isolation and Structure elucidation of the major degradation products of cefaclor in the solid state. *J Pharm Sci* 1997; 86: 540–9.
106. Dubost D, Kaufman M, Zimmerman J, et al. Characterization of a solid state reaction product from a lyophilized formulation of a cyclic heptapeptide. a novel example of an excipient-induced oxidation. *Pharm Res* 1996; 13: 1811–14.
107. Angberg M, Nystroem C, Castensson S. Evaluation of heat-conduction microcalorimetry in pharmaceutical stability studies: VII. Oxidation of ascorbic acid in aqueous solution. *Int J Pharm* 1993; 90: 19–33.
108. Oliyai R, Lindenbaum S. Stability testing of pharmaceuticals by isothermal heat conduction calorimetry: ampicillin in aqueous solution. *Int J Pharm* 1991; 73: 33–6.
109. Tan X, Meltzer N, Lindenbaum S. Solid-State stability studies of 13-cis-retinoic acid and all-trans-retinoic acid using microcalorimetry and HPLC analysis. *Pharm Res* 1992; 9: 1203–8.
110. Schmitt E, Peck K, Sun Y, Geoffroy J. Rapid, Practical and predictive excipient compatibility screening using isothermal microcalorimetry. *Thermochimica Acta* 2001; 380: 175–83.

111. ShalaeV E, ShalaeVa M, Zografi G. The effect of disorder on the chemical reactivity of an organic solid, tetraglycine methyl ester: change of the reaction mechanism. *J Pharm Sci* 2002; 91: 584–93.
112. Selzer T, Radau M, Kreuter J. Use of isothermal heat conduction microcalorimetry to evaluate stability and excipient compatibility of a solid drug. *Int J Pharm* 1998; 171: 227–41.
113. Selzer T, Radau M, Kreuter J. The use of isothermal heat conduction microcalorimetry to evaluate drug stability in tablets. *Int J Pharm* 1999; 184: 199–206.
114. Phipps M, Mackin L. Application of isothermal microcalorimetry in solid state drug development. *Pharm Sci Technol Today* 2000; 3: 9–17.
115. Mura P, Gratteri P, Faucci MT. Compatibility studies of multicomponent tablet formulations. *J Therm Anal Calorim* 2002; 68: 541–51.
116. Bruni G, Amici L, Berbenni V, Marini A, Orlandi A. Drug–excipient compatibility studies–search for interaction indicators. *J Therm Anal Calorim* 2002; 68: 561–73.
117. Wissing S, Craig D, Barker S, Moore W. An investigation into the use of stepwise isothermal high sensitivity DSC as a means of detecting drug–excipient incompatibility. *Int J Pharm* 2000; 199: 141–50.
118. Mroso P, Li Wan Po A, Irwin W. Solid-state stability of aspirin in the presence of excipients: kinetic interpretation modeling and prediction. *J Pharm Sci* 1982; 71: 1096–101.
119. McDaid F, Barker S, Fitzpatrick S, Petts C, Craig D. Further investigations into the use of high sensitivity differential scanning calorimetry as a means of predicting drug–excipient interactions. *Int J Pharm* 2003; 252: 235–40.
120. Harding L, Qi S, Hill G, Reading M, Craig DQM. The development of microthermal analysis and photothermal microspectroscopy as novel approaches to drug–excipient compatibility studies *Int J Pharm* 2008; 354: 149–57.
121. Airaksinen S, Karjalainen M, Kivikero N, et al. Excipient selection can significantly affect solid–state phase transformation in formulation during wet granulation. *AAPS Pharm Sci Tech* 2005; 6(41) (<http://www.aapspharmscitech.org>).
122. Morissette SL, Soukasene S, Levinson D, Cima MJ, Almarsson O. Elucidation of crystal form diversity of the HIV protease inhibitor ritonavir by high-throughput crystallization. *Proc Nat Acad Sci* 2003; 100: 2180–4.
123. Bauer J, Spanton S, Henry R, et al. Ritonavir: An extraordinary example of conformational polymorphism. *Pharm Res* 2001; 18: 859–66.
124. Taylor LS, Langkilde EW. Evaluation of solid-state forms present in tablets by Raman spectroscopy. *J Pharm Sci* 2000; 89: 1342–53.
125. Suryanarayanan R, Herman CS. Quantitative analysis of the active ingredient in a multi-component tablet formulation by powder x-ray diffraction. *Int J Pharm* 1991; 77: 287–95.
126. Lach JL, Bornstein M. Diffuse reflectance studies of solid-solid interactions. *J Pharm Sci* 1965; 54: 1730–6.
127. Weslowski M. Thermal methods of analysis in solid dosage technology. *Drug Dev Ind Pharm* 1985; 11: 493–521.
128. Byrn SE, Bugay DE, Tishmack PA. Solid-state nuclear magnetic resonance spectroscopy-pharmaceutical applications. *J Pharm Sci* 2003; 82: 441–74.
129. Markovich RJ, Anderson EC, Coscolluela CB, Zibas SA, Rosen J. Spectroscopic identification of an amorphous-to-crystalline drug transition in a solid dispersion SCH-48461 capsule formulation. *J Pharm Biomed Anal* 1997; 19: 661–73.
130. Chiou W, Rieglerman S. Pharmaceutical applications of solid dispersion systems. *J Pharm Sci* 1971; 60: 1281–302.
131. Chiou W, Rieglerman S. Pharmaceutical Applications of solid dispersion systems: x-ray diffraction and aqueous solubility studies on griseofulvin-polyethylene glycol 6000 systems. *J Pharm Sci* 1977; 66: 989–91.
132. Ford J, Rubinsten M. Ageing of indomethacin-polyethylene glycol 6000 solid dispersion. *Pharm Acta Helv* 1979; 54: 353–8.
133. Waterman KC, Colgan ST. A science-based approach to setting expiry dating for solid drug products. *Regulatory Rapporteur* 2008; 5: 9–14.
134. Tingstad J, Dudzinski J. Preformulation studies: II. stability of drug substances in solid pharmaceutical systems. *J Pharm Sci* 1973; 62: 1856–60.
135. Waterman K. Understanding and predicting pharmaceutical product shelf-life. In: Huynh-Ba K, ed. *Handbook of Stability Testing in Pharmaceutical Development Regulations, Methodologies, and Best Practices*. 1st ed. New York: Springer, 2009: 115–35.

136. Carstensen JT, Osadca, M., and Rubin, S. Degradation mechanisms for water-soluble drugs in solid dosage forms. *J Pharm Sci* 1969; 58: 549–53.
137. Tardif R. Reliability of accelerated storage tests to predict stability of vitamins (A, B₁, C) in tablets. *J Pharm Sci* 1965; 54: 281–4.
138. Carstensen JT, Rhodes C. Stability of solids and solid dosage forms. *J Pharm Sci* 1974; 63: 1–14.
139. Waterman KC, Adami RC. Accelerated aging: prediction of chemical stability of pharmaceuticals. *Int J Pharm* 2005; 293: 101–25.
140. Waterman KC, Carella AJ, Gumkowski MJ, et al. Improved protocol and data analysis for accelerated shelf-life estimation of solid dosage forms. *Pharm Res* 2007; 24: 780–90.
141. Monkhouse DC, Maderich A. Whither compatibility testing? *Drug Dev Ind Pharm* 1989; 15: 2115–30.
142. Carlson E. A high throughput approach for forced degradation and excipient compatibility studies. Princeton, NJ: Institute for International Research (IIR), February 24–25, 2004.
143. Zimmer A. CompaSys: Informations Sytem fur Galenische Vertraglichkeit. *Pharmazie* 1990; 135: 56–8.

12 | Small molecule parenteral drugs: Practical aspects of stress testing

Andreas Abend, Brett Duersch, and Kyle Fiszlar

INTRODUCTION

Parenteral drugs comprise a diverse class of medications that are not administered via the enteral (gastrointestinal) route. Parenteral drugs are typically targeted to provide systemic or localized exposure of the drug which may be achieved via injection, infusion, diffusion through the skin or mucous, or by inhalation or intravitreal application. This chapter on pharmaceutical stress testing will focus on small molecule parenterals which are administered to the patient in solution either intramuscular or intravenous. The principles outlined in this chapter may generally apply to all other routes of parenteral administration of small molecule drugs.

As for all drugs, parenteral drug products must be safe and efficacious. In addition, the drug products must be generally convenient for both the physician administering the product as well as the patient receiving the product. While safety and efficacy are typically demonstrated in animal and human studies, formulation development has to ensure that the final dosage form remains safe and efficacious over the shelf-life of the product.

Physical attributes, for example osmolality and viscosity, which may not be a patient safety or appearance concern also have to remain within specifications over the shelf-life of the product. In cases where the drug product is further diluted or reconstituted in a diluent prior to administration to the patient, the drug needs to be chemically stable in the dosing vehicle to ensure the safety and efficacy through administration.

Drug products in solution may or may not be stable for a prolonged period of time. Therefore, injectable drugs can either be formulated and provided to the physician as a solution or be stored as a solid and reconstituted in the dosing vehicle prior to administration.

This chapter will provide examples of pharmaceutical stress testing of small molecule drug products formulated as either a solution or a lyophilizate. Case studies presented in this chapter highlight specific concerns of drug product stability encountered during the development of these drug products and may or may not apply to the stability risks associated with the development of similar drug products. General considerations of pharmaceutical stress testing of drug products that may also apply to small molecule parenterals can be found throughout this book and the reader is encouraged to refer to chapters 1–4, and 19. Additional experiments performed over the course of the development of drug products A through D, which provided additional information about their specific product quality risks, are listed in Table 1.

PARENTERAL DRUG PRODUCT STRESS TESTING—CASE STUDIES

Water-Soluble Drugs

Stable Solutions: Case Study 1. Excipient and Leachable Impact on Product Photostability

The development of a drug product as an aqueous solution implies that the drug product is highly water soluble and stable in the proposed dosage form when stored at room temperature. Excipient compatibility and accelerated stress testing were performed on formulation prototypes of drug substance A to guide formulation selection as well as analytical methods development. Stress testing included exposing formulation prototypes to heat, hydrogen peroxide, basic and acidic conditions. Photostability experiments conforming with ICH Q1B (5) were performed with the final market formulation. Drug substance A is not photosensitive and based on the UV-Vis absorption spectra of the individual excipients as well as the aqueous formulation, no photodegradation of the drug product was expected. However, when the formulation was exposed to full ICH Q1 B confirmatory photostressing conditions (i.e., 200 W hr/m² of UV-A and 1.2 million lux hours of visible light), a number of degradation products were detected by HPLC (6). Analysis of the degradate peaks by HPLC-MS suggested a photo-induced hydroxyl radical-mediated degradation pathway.

Table 1 Pharmaceutical Stressing Experiments Performed in Addition to Standard Tests Typically Implemented During Drug Product Development for Drug Products A–D

Forced Stress Testing Base		Testing Required Beyond the Base			
		High-Water Solubility Drugs			Low-Water Solubility Drugs
		Case Study #1	Case Study #2	Case Study #3	Case Study #4
		Formulation Is a Solution	Formulation Is a Lyo Cake	Formulation Is a Lyo Cake	Formulation Is a Suspension
		Drug A	Drug B	Drug C	Drug D
Heat	20% RH	None	Extend humidity range down to <5%	Extend humidity range down to <5%	1. Hydrolysis of excipients in addition to drug 2. 100–123°C to characterize terminal sterilization
	75% RH				
Fe-mediated oxidation		1. Role of other metals 2. Iron titration, ppb range 3. Leaching from glass	None	None	None
Photostress		1. Titration of light exposure 2. Light mapping in manufacturing areas	1. Titration of light exposure 2. IV simulated light exposure	None	None

Additional photostressing experiments were conducted with formulations exposed to increasing UV-A and visible light to assess the magnitude of photodegradation (Table 2) as a function of UV-A or visible light exposure. This information provides useful guidance to the exposure limits necessary during manufacturing and packaging, shelf-life storage, and patient in-use.¹ In addition to a multitude of low-level degradation products, the most prominent photodegrade identified was a phenol. As one can see from Table 2, the phenol degrade exceeded the 0.15% [ICH Q3R (7) suggested safety qualification limit for drug substance A] with 900 K lux exposure to visible light. Considering the intensity of ambient indoor light, which is typically around 1000 lux for a laboratory or clinical setting, these data indicate that drug product A formulations are moderately photosensitive and suggests that storage of the drug product inside secondary packaging is necessary to ensure an adequate product shelf-life.

Compared to the length of formal stability studies (8)—typically 12–36 month storage under ICH conditions—the duration of photostressing experiments, which only require examination of one representative batch, is relatively short. A typical option 2 photostability chamber can deliver up to 10 klux of visible light and 10 W/m² UV-A light; common option 1 photostability chambers (e.g., xenon lamp-based chambers) can deliver in excess of 150 klux

¹Patient in-use photostability will be addressed in the next case. When infusable drug products are further diluted with commercial diluents for injection the photo stability of the final drug product administered to the patient is also examined.

Table 2 Photostressing Experiments Using a Typical Prototype Drug Product A Formulation Under ICH Q 1B Confirmatory Photo Stressing Conditions (Samples Were Presented to Increasing Visible Light up to 1.2Lux Hours and then Exposed in 50W/m² Increments to 200W/m² UV-A. Sample Analysis Was Performed by HPLC) (6)

Exposure Condition		% of Component Found		
Visible (Lux Hours)	UV (W hr/m ²)	Drug A	Phenol	Total Degradation Products
0.3 million	0	101.3	<LOQ	<LOQ
0.6 million	0	101.0	0.10	0.10
0.9 million	0	100.6	0.21	0.33
1.2 million	0	99.3	0.31	0.81
1.2 million	50	98.8	0.40	1.13
1.2 million	100	98.3	0.48	1.35
1.2 million	150	97.4	0.60	1.58
1.2 million	200	96.0	0.71	1.86

Table 3 Lot ID, Age of the Lot A Testing and Levels Phenol Detected After 1.2 Million Lux Hours of Visible Light Exposure

Lot ID	Age of Lot at Testing	Phenol Levels
Batch one	23 months	1.2%
Batch two	13 months	0.4%
Batch three	3 months	0.1%

and 60 W/m² UVA. Hence, the duration of a ICH Q1B photostability study may take only about 1 week for an option 2 chamber and about 8 hours for an option 1 chamber. Control samples with protection from light allow accounting for any thermal degradation inside the photostability chamber. Over the course of several photostressing experiments with formulation A, it was found that the levels of drug substance A photodegradants were not only a function of exposure time (dose), but also correlated with the age of the samples (Table 3). In order to understand the unexpected photosensitivity of drug formulation A and the increased sensitivity of formulations with storage, experiments were carried out to identify the contributing factors.

Formulation A: Photo Sensitivity Contributing Factor

Excipients

Formulation A consists of water for injection (solvent), NaCl (isotonicity), sodium citrate (buffer), and drug substance A. The pH of the formulation is pH 6.8.² One factor at a time experiments were carried out to identify the root cause to the photodegradation of the drug substance. Prototype formulations were prepared in the lab with varying excipient concentrations and / or excluding excipients. The result of the study clearly indicated that the photosensitivity was linked to the presence of citrate. Citrate alone is not known to induce photochemical reactions; however, citrate is known as a chelating agent for di- and trivalent metal ions. Di- and trivalent transition metal-ion citrate complexes in aqueous solutions are known to have both UV and visible light absorption (9). Heavy metals including transition metals are usually well controlled in pharmaceutical formulations; however, ppm levels of iron are common in excipients,

²The impact of pH on photodegradation was also studied. Keep in mind that infusible drug products are typically formulated at pH 4–8 for patient safety and convenience reasons.

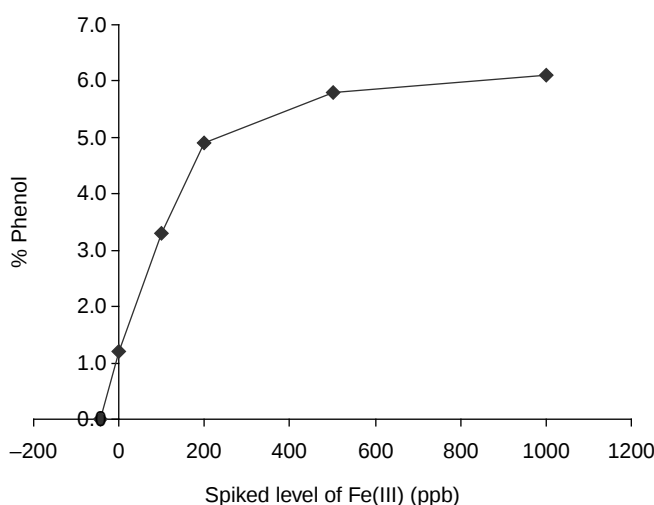


Figure 1 % Phenol degradation formation in formulations spiked with various amounts of Fe^{3+} with 1.2 million lux hours visible light exposure.

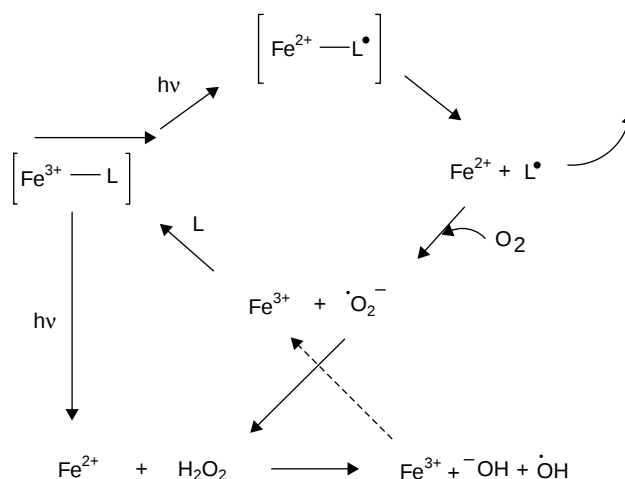


Figure 2 Photoinduced ferrous (Fe^{2+}) iron formation in solutions containing ferric iron (Fe^{3+}) and citrate leading to hydrogen peroxide formation (10). Bottom: Reduction of hydrogen peroxide by Fe^{2+} producing hydroxyl radical.

most drug substances, and can potentially be introduced via the manufacturing process and packaging components. In addition to citrate, the experiments demonstrated that dissolved oxygen and iron are also required to cause drug substance photodegradation when the formulation is exposed to light. With fixed light exposure, increasing ferric iron levels in the formulation over a range of 10–200 ppb showed linear degradation of drug substance A (Fig. 1).

Likewise, at controlled levels of ferric iron, the rate of drug substance A photodegradation was linear with light exposure. Samples sparged with helium to replace oxygen showed significant reduction in photodegradation compared to unsparged samples. Drug substance A photodegradation can thus be explained via the following mechanism involving iron, citrate, oxygen, and light (Fig. 2):

Fe^{3+} and citrate form inorganic water-soluble complexes. These complexes absorb light and undergo intracomplex reduction of Fe^{3+} to Fe^{2+} . Ferrous iron—either in complex with citrate and/or coordinated with water molecules—reduces dissolved oxygen thus generating superoxide radicals and Fe^{3+} which, in the citrate complex, can be photoreduced again. The superoxide is transformed via a series of reactions to hydrogen peroxide. Hydrogen peroxide is reduced by Fe^{2+} to yield hydroxyl radicals and hydroxide in a reaction known as “Fenton’s reaction” (11). The hydroxyl radical then abstracts hydrogen atoms from the drug substance. Hydroxyl radicals are highly reactive and are known to be capable of abstracting hydrogen atoms from primary, secondary, and tertiary C–H bonds. Therefore, the photodegradation mechanism explained here for drug substance A is also problematic for formulations containing iron (as a trace impurity), polycarboxylic acids, and dissolved oxygen.

Formulation A: Photosensitivity Contributing Factor

Leachables

Leachable studies are typically performed on parenteral drug products as part of formal stability studies. With parenteral drug products stored in glass vials and capped with a rubber closure, stability samples are usually stored in different positions (up-right, on the side, and inverted) to probe for the potential of leachables coming from the stopper closure. No difference in stability was observed with samples stored, inverted, and up-right positions during routine analysis of stability samples stored under ICH conditions (8).

Aged samples of formulation A were analyzed by ICP-MS and showed increased levels of Si, Al, Ca, Ba, and Fe—all components of the borosilica glass vial used as primary package for the drug product. The ICP-MS data together with the iron spiking experiments explain effectively why aged batch samples of drug product A are more photosensitive than fresh ones. The data also further manifest that iron is the trace metal that in the presence of citrate, oxygen, and light is capable of inducing the hydroxyl radical-mediated drug degradation.

Based on the experiments outlined above, the extent of drug formulation A degradation is directly proportional to the amount of dissolved iron and light exposure. In order to assess the product-quality risks associated with photodegradation during manufacturing and packaging operations, one must know maximum levels of iron in the formulation introduced via the excipients and the time and intensity of the light that the formulation is exposed to over the course of the manufacturing and packaging process. The maximum levels of degradation products acceptable for a given drug product are either based on safety qualification limits following ICH Q3B (R2) or established through additional safety studies. The ability to meet these specifications over the shelf-life of the product will dictate the need for additional controls of iron or light exposure during the manufacturing and packaging operations as well as addition for the product label to “store in secondary package.”

Unstable Solutions—Lyophilized Drug Products

Freeze-dried parenteral drug products are usually developed to stabilize moisture-sensitive water-soluble drugs (12). Common critical quality attributes of a lyophilized drug product can be found in the literature and the appropriate compendia (1,12,13) and include completeness and clarity of the reconstituted solution, assay and degradates, content uniformity, pH, endotoxins, sterility, particulate matter (visual and HIAC), osmolality, and others identified during development such as moisture for lyo products or tests specifically required by regulatory agencies.

The concerns with the development of two lyophilized drug products with remarkably different stability properties will be discussed below. The focus will not be on the routinely performed excipient compatibility or formal stability testing in accordance with ICH guidelines but rather will be on outlining a more holistic scientific approach to ensure that adequate controls are in place during the manufacture, storage, and patient in-use of these two drug products.

Case Study 2: Drug A, Light Cake Weight, Sensitive to Moisture and Light**Chemical Stability of Drug A**

Drug A was developed for both acute and chronic treatment of patients with respiratory illnesses. An IV form of the drug was desired to treat patients suffering from acute respiratory issues in a hospital emergency room setting. Early in development, the battery of testing described in the literature (14–19) was employed to assess the stability of the drug substance as well as the various drug product formulation candidates. The instability of the drug in an aqueous environment led to the need to introduce a lyophilized dosage form to generate the desired 2 years shelf-life for the commercial utility of the product. The key degradation pathways for this drug molecule were identified as oxidative degradation and photodegradation (Fig. 3) (20).

Formulation development efforts for the lyophilized drug revealed that oxidative degradation can be effectively mitigated by using appropriate excipients (21) and by limiting exposure to oxygen during the manufacturing process as well as upon storage (12,22). Photostability experiments (4,23) with the drug product exposed to ambient laboratory light demonstrated the sensitivity of the drug towards photoisomerization especially when in solution (24). The extreme photosensitivity of the drug was successfully addressed by performing all manufacturing operations under filtered (yellow) light and during long-term storage by packaging the lyo-product in a light-resistant secondary package. In contrast to the high sensitivity to light, oxidative degradation was negligible during manufacture, reconstitution, and patient use. To mitigate the risk of oxidation upon storage, the drug product was lyophilized, blanketed with dry nitrogen, and closed with a bromo butyl rubber closure.

Closure integrity was established by measuring the head-space oxygen content as well as microbial testing performed during formal stability studies. As far as microbial limits testing is concerned, there are now more reliable and rapid tests for closure integrity testing available. These include, for example, residual seal force (25), vacuum decay, and dye ingress (26).

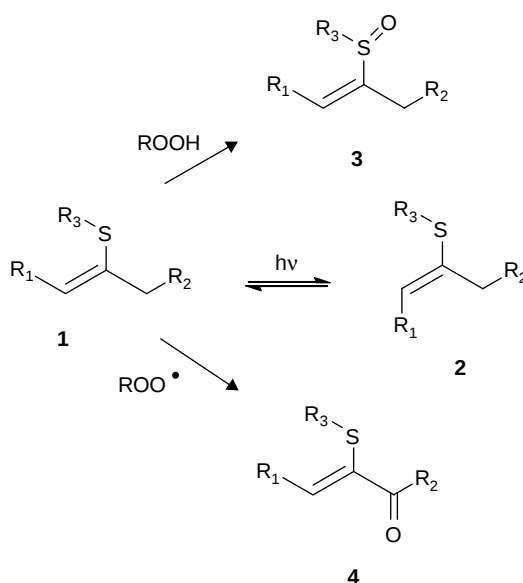


Figure 3 Degradation pathways of drug molecule A: Photoisomerization of isomer A (1) to isomer B (2) is caused by exposure to light. Organo hydroperoxides react with 1 at the sulfur atom affording sulfoxide 3. Hydroperoxy radicals transform 1 to ketone 4. Note that isomers of 3 and 4 can be formed from isomer B (2).

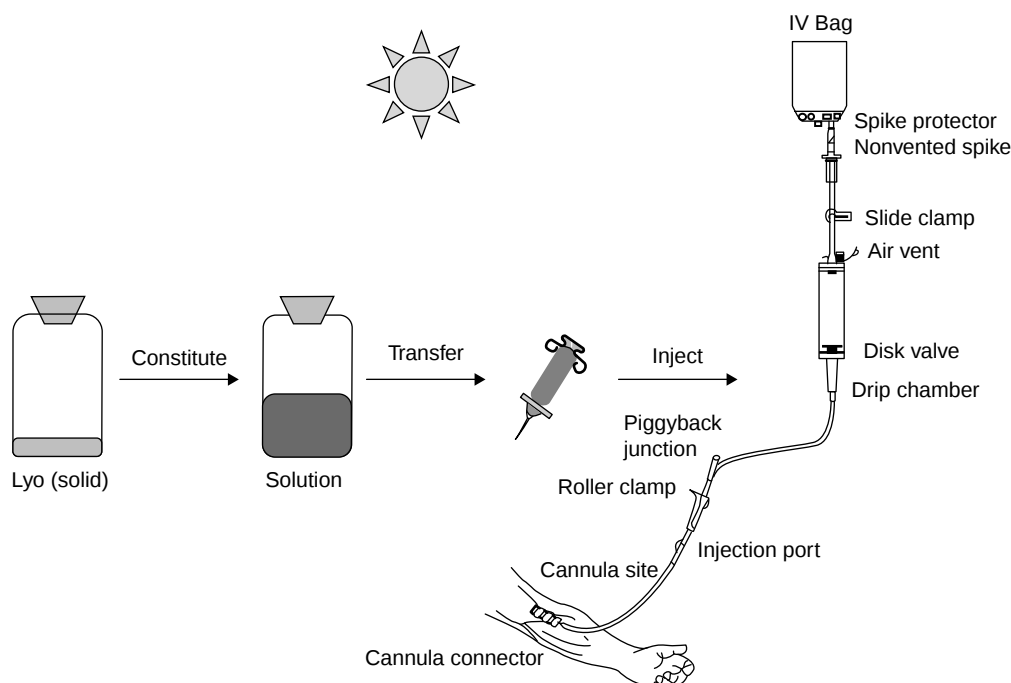


Figure 4 Schematic of patient being dosed with product A.

Patient In-Use Stability

Parenteral drug products formulated as a solution are either injected directly or they may be infused via open ports of existing IV lines. Other injectables are diluted in a commercial or custom-made aqueous diluent and then administered via a drip bag. Before they can be administered to the patient, lyophilized drug products require constitution in a commercial or custom diluent. Figure 4 gives an overview of the steps required to prepare a lyophilized drug product for patient administration.

Compatibility of the drug product with commercial diluents (27) is typically verified by combining the drug product (or the constituted drug product in the case of lyo products) with the required amount of diluent and evaluating assay and degradation products as well as visual changes including turbidity and particulates. Small particulates invisible to the eye are typically measured via light obscuration, light scattering, and or microscopy. These studies are usually performed over a 24 hours period of time to gain confidence that there are no adverse interactions with the commercial diluent.

Lyo product A posed an interesting problem with respect to diluent compatibility. The drug substance has excellent solubility in water at concentrations $>5\text{ mg/mL}$; however, at concentrations below 1 mg/mL , the drug showed precipitation. Furthermore, the drug product solubility was sensitive to the pH and sodium concentration of the commercial diluent. Table 4 shows the commercial diluent constitution experiments with lyo product A. As the table indicates, the constituted drug product is only stable in diluents containing dextrose and relatively low sodium chloride concentrations. Supplying the drug product worldwide poses a challenge since some of the diluents tested were not available to all markets.

Photostability of Drug Product A

The *cis/trans* isomerization (Fig. 3) of the drug substance is driven by the direct absorption of light by the drug molecule. The causative absorption wavelengths (Fig. 5) of concern are

Table 4 Compatibility Results of Drug Product A with Commercial Diluents (Drug Degradation and Assay Was Not an Issue in Any of the Media; However, Precipitation or Gelling of the Drug Was Observed in Several Media which Is not Acceptable from a Patient Safety Perspective)^a.

Vehicle	Supplier ^a	Acceptability	Comment
Sterile water for injection	Worldwide	No	Stable solution but hypotonic
0.9% saline	Worldwide	No	Hazy solution formed immediately
0.45% saline	Worldwide	No	Hazy solution formed immediately
5% dextrose	Worldwide	No	Precipitates within minutes
2.5% dextrose	Worldwide	No	Hypotonic
1.25% sodium bicarbonate	Worldwide	No	Hazy solution formed immediately
3.3% dextrose/0.3% saline	Abbott; Baxter (CAN)	Yes	Stable for 24 hrs
3.3% dextrose/0.225% saline	Abbott; Baxter (CAN)	Yes	Stable for 24 hrs
5% dextrose/0.33% saline	Abbott; Baxter (US)	Yes	Stable for 24 hrs
5% dextrose/0.2% saline	Abbott; Baxter (US)	Yes	Stable for 24 hrs
5% dextrose/0.45% saline	Abbott; Baxter (US)	No	Hazy solution formed immediately
2.5% dextrose/0.45% saline	Abbott; Baxter (US)	No	Hazy solution formed immediately
Ringer's solution	Worldwide	No	Hazy solution formed immediately
Lactated Ringer's solution	Worldwide	No	Hazy solution formed immediately

^aThis assessment was conducted in the late 1990s.

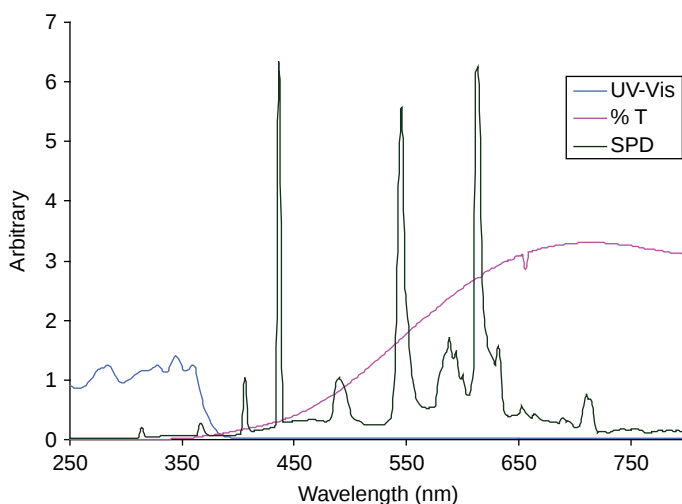


Figure 5 UV-Visible absorbance spectrum of drug product A, transmission of USP-type amber flint glass, and the SPD of cool white fluorescent light bulbs commonly used in manufacturing areas (29).

those above 300 nm since most indoor light sources (including glass filtered day light) emit radiation in this region. As Figure 5 shows, the drug molecule absorbs light in the UV region up to 400 nm. The spectral power distribution (SPD) of low-pressure mercury fluorescent light bulbs found in most manufacturing areas reveals mercury emission lines at 313 and 365 nm that overlap with the UV-Vis of the molecule and thus are capable of inducing photodegradation (28). Typical light intensities in manufacturing areas and testing labs are ~800–1000 lux. Similar SPDs as shown in Figure 5 were found in four local Philadelphia hospital emergency rooms during an assessment of potential patient in-use stability concerns

with drug A. While the SPD measured in hospitals revealed the predominance of low-pressure mercury fluorescent light bulbs, the light intensities at the site of administration varied significantly from 300 to 2000 lux.

There are several strategies that can be utilized to minimize light exposure at wavelengths in the 300–400 nm range region that are effective to minimize the overall photodegradation of the product under the various exposure conditions. Nonetheless, consideration of light exposure during manufacturing, packaging, storage, and drug administration are required for photosensitive drug products (29). The total degradation levels must then be considered against safety qualification criteria. Ultimately, one must decide where the light exposure-driven photo degradation levels are best allotted to ensure the safe and efficacious use of the product. The strategy detailed below focuses on minimizing photodegradation during manufacturing, packaging, and storage, thus maximizing drug administration flexibility.

Drug product A was lyophilized into USP-type amber flint glass vials which have a light transmission profile (%T) as shown in Figure 5. The *y*-axis of this plot was scaled for illustrative purposes and does not reflect the actual units of measure. Typical %T of the amber glass was 50% at 700 nm. The absorption maximum of drug product A at a concentration of 0.02 mg/mL in 50% methanol/water is 2 absorption units from 200 to 360 nm. Transmission and SPD measurements were performed with the OL-754 from Optronics Lab Inc.

CONSIDERATION OF LIGHT EXPOSURE FOR THE DRUG PRODUCT

Manufacturing and Packaging

The overall development and manufacturing of lyophilized drug products is effectively explained in chapter 9. A systematic analysis of the drug manufacturing process at the final manufacturing and packaging sites revealed the following potential areas for light exposure:

1. drug substance weighing;
2. filling of the vials and potential hold-up in the filling lines;
3. storage of the filled vials prior to charging the lyo chamber;
4. storage of the vials post lyophilization;
5. inspection;
6. storage of the vials post inspection and prior to labeling and packaging;
7. packaging.

From photostability studies performed during drug product development (29) evaluating the drug substance, the fill solution, and the lyo cake, it was concluded that manufacturing and packaging steps had to be performed under protection from light. As apparent from Figure 5, the lyo product is still light sensitive in the amber vial under ambient conditions when stored for extended periods of time (i.e., several weeks). To minimize any further photoisomerization, the inspected drug product was therefore shrouded in black plastic foil before final packaging in the secondary packaging. ICH Q1B confirmatory photostability testing has shown that the product will undergo no photodegradation when stored inside the secondary package.

Drug Product Administration

The lyophilized drug will be shipped to the hospital in the secondary package. Labeling instructions will indicate that the product will have to remain inside the package until reconstituted for administration. The three proposed steps (Fig. 4) during the overall drug administration process where photodegradation can occur were identified as follows:

1. reconstitution of the lyo cake with diluent and holding of the reconstituted product in the amber vial;
2. transfer of the solution into a clear glass syringe and holding of the reconstituted solution in the syringe;

3. infusion of the drug into the patient through an existing IV line or direct infusion of the solution as a bolus over a given period of time. Due to safety concerns, the drug product was infused within 5 minutes.

Additionally, evaluation of the lighting (SPD and illuminance levels) present in the room(s) where the drug is reconstituted and administered is important to complete a proper assessment of the overall extent of potential photodegradation.

Representative Lighting Conditions in Hospital Emergency Care Units

Emergency rooms in four local Philadelphia area hospitals were visited and the light sources and level of intensity were assessed. All emergency rooms were illuminated by white fluorescent light bulbs, with brightness levels varying from 250 to 2100 lux. As explained for the manufacturing process above, fluorescent light contains emission wavelengths below 400 nm and thus will induce drug photodegradation of drug product A.

Representative Exposure Points During Product Reconstitution and Administration

The drug product will potentially experience light exposure as (i) outside the secondary package, (ii) as a reconstituted solution in the primary package, (iii) as a reconstituted solution in a clear glass syringe, and (iv) in the IV administration tubing (Fig. 4). Note that drug product A was likely administered into an open port of an existing IV tubing line. The compatibility of the reconstituted solution with commercially available diluents that may be present in the IV tubing was established in experiments similar to those described in the literature (30).

The main apparent mechanism of photodegradation observed for the drug product reconstituted solution is the *cis/trans* isomerization shown in Figure 3. *Cis*-isomer formation rates have been determined in reconstituted solutions stored in amber vials, under the “worst case” hospital light exposure conditions noted above (i.e., 2000 lux) as a function of exposure time. No photodegradation is expected in syringes completely wrapped in aluminum foil. It is important to note that the degradate ICH qualification threshold is 1.0% or 50 µg (whichever is lower) for drugs with a maximum daily dose of <10 mg. Finally, the overall time from reconstitution to completion of administration is limited to 1 hour for this assessment.

The overall estimates of levels of isomer B formation in each stage of product administration is represented in Figure 6, employing the assumptions stated above, and using a clear glass syringe and IV tubing with no over wrap for light protection.

Lyophilized drug product A in the amber vial is stable for days exposed to fluorescent light at <2000 lux. Negligible amounts of photodegradation of the reconstituted solution are anticipated inside the amber vial over several hours. The main photodegradation occurs in the unprotected syringe, and thus the exposure time of the unprotected syringe is the most critical. Strictly following the labeling instructions would produce isomer B levels exceeding the ICH qualification levels even when administered over the recommended 5 minute time period. Note that direct bolus injection within 2–3 minutes without an IV line would probably be acceptable with a clear syringe.

As Figure 6 shows, administration of drug A could potentially expose patients to unacceptable levels of isomer B. Even though the labeling instructions may be clear and very specific, unintentional exposure of the reconstituted drug in the syringe for prolonged periods of time cannot be completely ruled out. Therefore, either foil wrapping the syringe or the use of amber syringes might be considered to mitigate this risk.

Physical Stability of Drug A

Drug substance A is provided as a crystalline material but is dissolved and then re-dried to an amorphous form during lyophilization. The physical state of drug product A was monitored over the shelf-life and shown to remain amorphous. Although conversion into a crystalline form

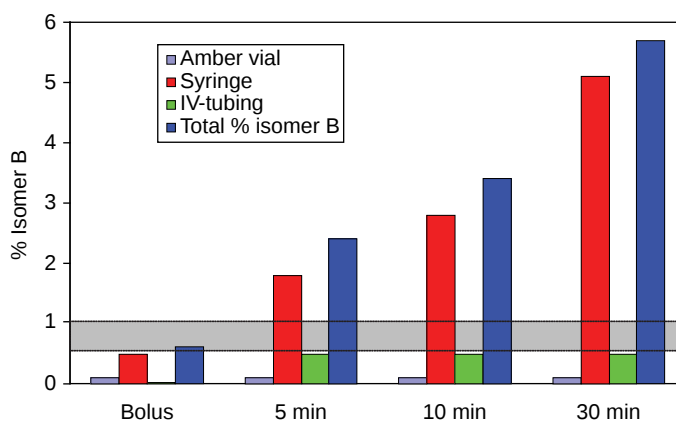


Figure 6 Estimated levels of photoisomerization during drug administration. Reconstituted drug product A was exposed to 2000 lux visible light inside an ES 2000 photostability chamber. Samples were presented in the amber vial (turquoise bars) and in clear glass vials (red bars) to simulate degradation in glass syringes. Photodegradation experiments in the IV tubing (green bars) were performed by passing the reconstituted solution through a 30 cm IV tubing exposed to 2000 lux fluorescent light over 5 minutes. Shown here is also the ICH qualification limit for drug product degradates in finished products. Depending on the actual dose, the qualification limit can be 0.5 or 1.0% (gray band).

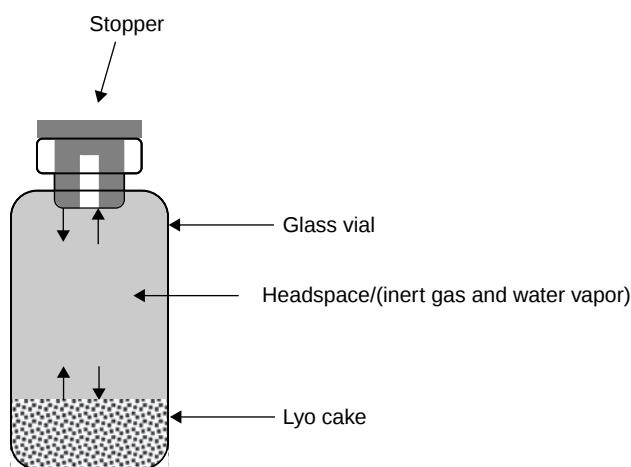


Figure 7 Typical glass vial/stopper configuration. Shown here is a slotted stopper on top of a glass vial with the lyo cake on the bottom and gas in the headspace. The arrows indicate exchange of gases/water vapor from the cake through the headspace and the stopper.

could manifest itself in cake shrinkage, the known polymorphs of drug A were shown to be highly soluble in diluents containing dextrose and NaCl which were specified for reconstitution of the lyo cake.

Drug A lyophilizes as a voluminous, white, amorphous cake. Microscopic images of the cake revealed a vast network of pores and channels that are indicative of a large surface area. Because of the high intrinsic volume of the light cake, rapid equilibrium with the environment was a concern. As a benefit of the lyophilization process, exposure to atmospheric oxygen and moisture can be effectively controlled since post freeze-drying the vials can be either capped under vacuum or the chamber can be completely or partially filled with an inert gas prior to pushing the stoppers into the vial. Once the stoppers are crimped on the vial, the system can be regarded as a closed system with minimal material exchange with the outside environment (Fig. 7) (31).

Since drug A is fairly chemically stable in the absence of oxygen and light, the main concern was controlling moisture. Adequate cake moisture during freeze-drying can be monitored via temperature sensors at the vial trays and by monitoring water content in the lyo chamber by mass spectroscopy or, alternatively, after sealing the vial with water-specific methods (1). Post flushing of the lyo chamber with an inert gas and stoppering the vials, the main source of moisture comes from the stopper closure (32–34) (the glass is usually not a sink of moisture and the diffusion of water from the outside through the glass wall and the stopper can be considered negligible relative to the shelf-life of the product) (35). Figure 7 shows that the moisture sources contributing to the total moisture in the vial stem from the residual moisture left in the cake, the initial moisture in the vial headspace at seal closure, and moisture available from the closure (36).

The moisture sorption isotherm of the lyophilized cake revealed a hysteresis indicative of irreversible changes (i.e., visually observable cake collapse) when the cake moisture reaches ~14% w/w. With drug product A, the cake will reach 14% w/w of moisture when exposed to a 35% RH environment. To put this into perspective, in order to achieve 1.4 mg (equivalent to 14% w/w of water) in a 10 mg cake and 35% RH in a 30 mL vial (that is 0.24 ng of water—assuming the cake volume is negligible), the total amount of water available to the cake and headspace has to be ~2 mg (37,38). Assuming that the fill gas and the cake immediately after lyophilization are completely dry, which for lyo cakes is not practical nor necessary, theoretically 2 mg of moisture would have to reach the vial inside either through improper closure integrity or coming from the stopper (31). The stopper will also be in equilibrium with the headspace—a very dry stopper could absorb water from the headspace while a “wet” stopper could release water into the headspace. A 20 mm stopper is suitable for a 30 mL vial and weighs about 2 g. Typical moisture levels found with bromo butyl stoppers as received from the manufacturer are around 0.7% water (39) equivalent to 14 mg. Even though it is apparent from Figure 4 that in a stopper where moisture is distributed homogeneously not all of this moisture can possibly reach the cake over a realistic shelf-life (33), at least parts of the stopper exposed to the inside of the vial will equilibrate with the cake and inert gas and change the initial headspace moisture until the system reaches equilibrium. This equilibrium assumes no water travels through the stopper within the discussed time.

For drug product A, one of the most critical process parameters related to ensuring adequate lyo cake appearance was the stopper drying process (39–41).

Figure 8 shows the amount of residual water tested by KF in 20 mL bromo butyl rubber closures as received, after steam sterilization with no drying, and with drying at 115°C in a convection oven for 1–10 hours.

Figure 8 shows the amount of moisture absorbed by a 10 mg lyo cake with stoppers dried for varying durations at 115°C (bars in blue). As one can see, the total amount of moisture left in the stoppers does not significantly change with drying times >1 hour; however, the amount of water available to the cake does significantly change (magenta bars). The lyophilized drug product showed cake shrinkage and collapse when stored with stoppers dried for 2 hours, whereas stoppers dried for 4 hours and more showed negligible evidence of physical change over 13 weeks stored at 40°C. The headspace moisture in the vials was determined prior to opening them for cake moisture measurements. The bars in yellow demonstrate a strong correlation between the cake moisture and headspace moisture which is consistent with the equilibria established inside the vials.

Examination of the vials stored at 40°C at earlier time points showed that for vials closed with stoppers “as received”, autoclaved, and nondried, dried for 1 and 2 hours, etc., the vials with the autoclaved and nondried stoppers showed cake shrinkage and collapse early on. Vials closed with stoppers dried for 1 hour showed cake shrinkage followed by collapse over 3 weeks. Lyo cakes inside vials closed with stoppers dried for 2 hours and “as received” showed shrinkage after 13 weeks and when closed with stoppers dried for >2 hours, no shrinkage or signs of collapse were observed after storage for 13 weeks at 40°C.

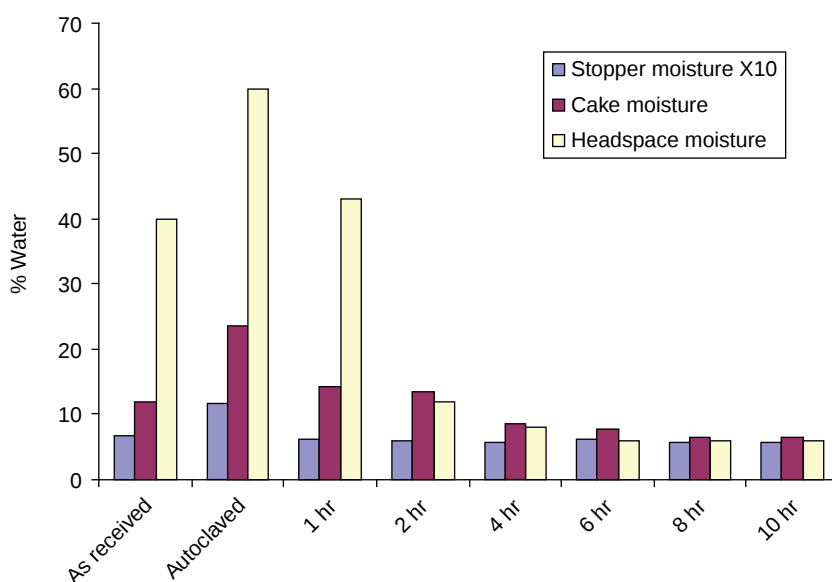


Figure 8 Stopper moisture in stoppers “as received,” steam autoclaved, and dried at 115°C for several hours was measured by KF and results are shown in blue (39). The w/w% water content was multiplied by 10 for illustrative purpose. Cake moisture was also measured by KF and data (w/w%) are shown in magenta. The stoppered lyo product A samples were stored at 40°C for 8 weeks and the headspace moisture was determined by laser frequency-modulated laser spectroscopy (42) after 8 weeks.

The results from this study can be explained by the distribution of water in the stoppers (31,36). An as-received stopper will likely be completely equilibrated with the storage environment. In this case, all water in the stopper may be distributed throughout the stoppers. When the stoppers are steam autoclaved and subsequently dried for relatively short periods of time, this equilibrium is disturbed. It is plausible that most of the moisture introduced during steam autoclaving is adsorbed at the stopper surface and migrates into the stopper. The distance the water penetrates into the stopper will depend on the temperature, the pressure, and the properties of the stopper material which impact the water diffusivity (Fig. 9). Once the stoppers are dried, moisture is removed primarily from the stopper surface. The rate and extent of surface moisture removal during the drying step is dependent on the temperature, the humidity in the dryer, and drying time. While stopper moisture measurements by KF determine the residual moisture of the entire stopper, these measurements do not assess the moisture sitting at the stopper surface. This may explain why stoppers as received and stoppers dried for 1 hour or 2 hours show very different cake behavior when stored for 13 weeks. As mentioned above, the stoppers directly taken from the steam autoclave will cause rapid cake collapse—here ample moisture on the stopper surface will be rapidly available to the dry cake and headspace. Stoppers dried for 1 hour may not have much residual surface moisture, and water vapor from the inside of the stopper will have to travel through the stopper before it enters the vial. Stoppers dried for 2 hours will contain even less surface moisture and the water contained in the stoppers will have to travel even farther from the inside to the stopper surface before it is available to enter the vial. KF moisture measurements of stopper closures in the case of drug product A show that the sensitivity and accuracy of the measurement are not sufficient to discriminate between acceptable and nonacceptable stopper dryness. The data collected on stoppers dried for 1 hour, for 2 hours, and longer all have comparable water contents; however, they show very different cake performance as shown in Figure 8.

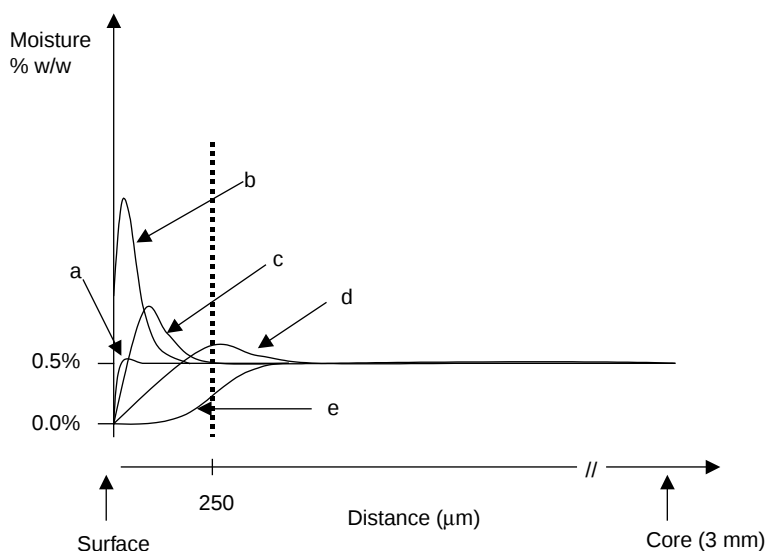


Figure 9 The distribution of water in rubber stopper closures (36). Shown here are the hypothetical distributions of moisture inside a stopper from the stopper surface. A hypothetical 250 μm distance—estimated from a diffusivity constant (33) of $\sim 4 \times 10^{-11}$ m/second indicates how far moisture may have to travel from the stopper core to the surface in order to be released into the vial. Trace (a) shows the moisture distribution in an as-received stopper with most of the moisture distributed evenly across the stopper. In this case, a small amount of surface moisture may have been removed from the stopper surface during the lyo process. Trace (b) shows that with autoclaving water is “loaded” into the stopper and the amount of moisture at the surface after lyophilization is much higher compared to the as received and dried stoppers. Trace (c) shows that some of the moisture which entered the stopper during autoclaving has moved further into the stopper and the distance the moisture has to travel to reach the surface and the inside of the vial has increased. Trace (d) continues to show this trend with prolonged drying where as trace (e) stipulates that stoppers dried for prolonged amounts of time may never reach the surface over the shelf-life of the product.

This study effectively highlights the need to gain adequate knowledge about stopper drying conditions. While for drug A only cake collapse was a quality concern, chemical stability as well as other physical changes that might compromise patient safety or product performance (i.e., time for reconstitution) must be carefully studied. Since the data obtained in early development was from small scale and therefore ideal drying conditions such as monolayer drying in a small uniform dryer, careful stopper drying studies at full process scale are required to ensure adequate drying with stoppers autoclaved in bags and dried in large multitrack driers that may have different heating zones and heat convection properties. This study also effectively demonstrates that measuring stopper dryness by traditional KF can be misleading and headspace moisture in the vial is more indicative of potential product change.

Case Study 3: Chemical Instability of Drug Product B

Unlike drug A, which was chemically stable in aqueous media over several days and also stable in most diluents for injections, early stability studies demonstrated the propensity of drug B to form insoluble degradants via hydrolysis in the pre-lyo solution, upon storage as a lyo product, and after reconstitution prior to administration to the patient.

Pre-lyophilization hydrolysis was effectively reduced by controlling the solution pH and temperature of the solution. Adjustment of the pre-lyo solution pH also had a profound impact on the rate of hydrolysis upon storage. Other factors contributing to drug B hydrolysis upon storage were identified as overall cake moisture and the presence of iron. In response to these

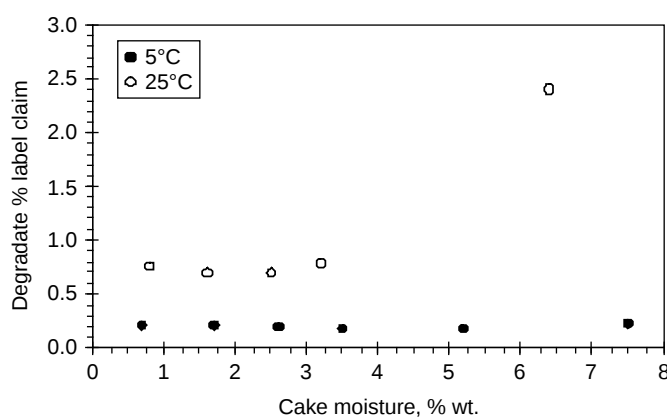


Figure 10 Effects of temperature and moisture on the formation of the insoluble degradate in drug B.

sensitivities, the formulation was carefully designed to maintain the appropriate pH and to chelate metals. Additionally, a bulking agent was chosen to maintain a high glass transition temperature in the final lyophilized product to further slow the rate of hydrolysis.

The sensitivity of the drug to water required the production of a lyophilized product to provide the desired shelf-life stabilities by minimizing hydrolysis within the drug product. As discussed in case study 2, lyophilized drug product A, the sources of water available for chemical degradation in case study 3, lyophilized drug B, are residual water in the cake following manufacture, moisture in the fill gas, and water that can desorb from the stopper during storage.

The effects of moisture and temperature on drug product B were explored by exposing lyophilized cakes to steady-state moisture environments ranging from 5 to 40% RH at 5°C and 25°C. Such relative humidity conditions can be achieved with the use of pre-equilibrated silica or salts and/or their various hydrate forms that are known to produce distinct levels of head space moisture when kept in a closed environment such as a lab desiccator. The cake appearance, cake moisture, and product degradation were monitored through 6 months of storage at the two temperatures and multiple humidities. No change in cake appearance, typified by >2 mm physical shrinkage or total collapse, was observed for cakes stored at 5°C at water levels $\leq 5.2\%$ w/w and at 25°C at water levels at or <3.2% water. The degradate levels measured for these same samples are plotted versus lyophilized cake water in (Fig. 10). Note that for lyophilized cakes stored at 5°C with water levels at or <7.5% cake moisture and at 25°C with water levels at or <3.2% where cake collapse is not observed, chemical degradation is a function of temperature only, not cake moisture.

The data above are consistent with the glass transition temperatures reported in Figure 11 below for the high moisture conditions. Consistent with the Gordon–Taylor rule, the glass transition temperature drops as the cake moisture increases (43). Storage above the glass transition temperature affects the physical stability, cake appearance/collapse, and chemical stability. The effects are attributed to increased solid-state mobility promoting physical cake collapse and more rapid degradation. Note that considerable amounts of water are required to reduce the glass transition temperature under long-term storage and manufacturing conditions (>7.5% cake moisture at 5°C and >3.2% moisture at 25°C).

Overall, the data set allows the scientist to establish the critical moisture content at the studied temperatures (5°C and 25°C) to then allow a design space for the development of a product (i.e., appropriate package and process outcomes). Furthermore, a carefully developed lyophilization cycle and stopper drying process will then ensure that the moisture in the drug product will remain well below critical moisture levels where undesired physical and chemical changes can occur.

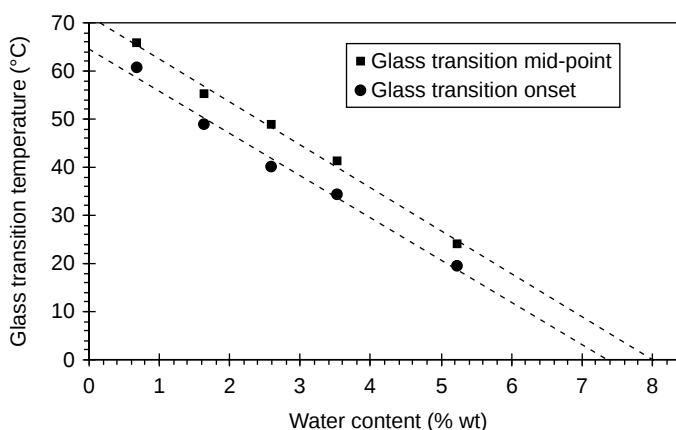


Figure 11 Impact of moisture on glass transition temperature.

PARENTERALS OF LOW-WATER SOLUBILITY DRUGS

Case Study 4: Infusible Drug Product Formulated in a Microemulsion

Drug R was developed for acute treatment of patients suffering from congestive heart failure. Early in development, the battery of testing described in (44) was employed to assess the stability of the drug substance as well as the various drug product formulation candidates. The API was demonstrated to be extremely stable when stressed with heat, light, acid, base and both free radical and molecular oxidation. However, due to the low aqueous solubility, the drug was formulated as a parenteral oil in water emulsion leading to physical stability as the primary concern over chemical stability for this product.

Commercially available lipid injectable emulsions are formulated as 10–20% oil-in-water dispersions. The emulsions are most commonly formulated using soybean oil as the sole source of oil, but mixed oil systems (safflower oil, medium chain triglycerides, or olive oil) are also possible. The robust nature of the drug toward oxidative degradation allowed considerable flexibility in formulation development without limitation of using generally oxidation promoting lipid systems. These oil-in-water dispersions are combined with emulsifiers in the form of mixed phospholipids typically derived from egg lecithin. The resulting coarse emulsion is then passed through a microfluidizer to disperse the emulsion into finer droplets enhancing the physical stability and lowering the potential for adverse events. Typically, these formulations are then terminally sterilized for sterility assurance (45,46).

Initial formulations of drug R were not physically stable upon terminal sterilization (121°C for 20 minutes) with respect to viscosity. The unsterilized emulsion possessed a viscosity of 1–2 cP which is equivalent to water. Upon terminal sterilization, the viscosity increased to ~40 cP. Storage of the material at both accelerated and long-term stability conditions caused a further rise in viscosity to >60 cP. While the viscosity of parenteral products is generally tolerated up to 200 cP, the stability study of the terminally sterilized material was discontinued due to the unexplained rise in viscosity.

As the development of the formulation and process continued, the physical stability improved and the severity of the viscosity change decreased. Since a significant change in viscosity using the standard terminal sterilization condition was observed, stability studies were initiated to evaluate the impact of varied temperature and time exposure on viscosity change consistent with the European Union decision tree on terminal sterilization (EMA Guidance CPMP/QWP/054/98, April 2000) (Table 5).

An evaluation was conducted on the impact of the various exposure conditions on viscosity of the product. Since an underlying mechanism for the change in viscosity was not able

Table 5 Impact of Thermal Sterilization on the Viscosity of Drug R Immediately after Sterilization and with Storage at 25°C

Temperature Exposure (°C)	Time Exposure (min)	Viscosity After Initial Exposure ^a	Viscosity After 8 Weeks	Viscosity After 11 Weeks	Viscosity After 22 Weeks (cP)
100	10	3.12	3.23	Not tested	3.43
108	10	3.12	3.30	Not tested	3.42
115	10	3.54	3.66	Not tested	3.83
115	20	4.21	4.75	Not tested	5.35
115	32	6.61	Not tested	7.52	Not tested
118	16	5.45	Not tested	6.93	Not tested
121	8	6.08	Not tested	7.31	Not tested
123	5	4.70	Not tested	5.70	Not tested

^aInitial values for all samples after aseptic processing were 2.21 cP.

to be determined, it posed a significant risk to the overall robustness of the formulation compared with the limited increase in sterility assurance that would be gained by a very gentle heat treatment cycle.

Additional Physical Concerns Related to an Emulsion

While emulsions require monitoring of standard physical and chemical tests such as assay, degradates, pH, osmolality, sterility, and endotoxins, the physical stability of the product is also monitored via droplet size and PFAT5 (percentage of fat molecules >5 µm) (47), which is not typically conducted on other parenteral formulations. Monitoring of the droplets contained within an emulsion is the subject of a recent addition to the general chapters of the USP (USP <729, Globule Size Distribution in Lipid Injection Emulsions>). The size of the droplets contained within the emulsion, and more specifically the number of large diameter droplets, have been linked to both the physical stability of the emulsion and to patient safety. A larger mean droplet size, and greater number of large droplets, increases the probability of coalescence leading to phase separation. Additionally, intravenous infusion of large oil droplets poses a significant risk of deposition on the lung leading to fibrosis and decreased lung function.

The process used to manufacture the emulsion is obviously critical to the droplet size distribution achieved in the final product. The rate, pressure, and temperature applied during micro-fluidization will directly affect both the mean droplet size and the PFAT5. The coarse emulsion (prior to microfluidization) will phase-separate over the course of a few hours without constant agitation while a well dispersed and micronized emulsion will be stable for at least 2 years. Ensuring a process that drives the emulsion to a well-micronized state is critical to achieving adequate shelf-life for a pharmaceutical product.

Once a stable emulsion has been achieved, the primary packaging is critical to maintaining the physical stability. The impact of the primary packaging on PFAT5 has been evaluated and it has been demonstrated that long-term storage of emulsions in glass containers is preferred over storage in plastic containers (48,49). The storage of emulsions in plastic containers causes a significant increase in the PFAT5 value leading to an increased risk of phase separation. While long-term storage of emulsions in glass vials or bottles is common practice, it is difficult to avoid contacting plastic components during dosing. Through transfers to IV bags, syringes, transfer lines, and other dosing components, the emulsion can remain in contact with these types of components for up to 24 hours. Due to differences in hospitals worldwide, the procedures used for dosing often vary from site to site. While compatibility with dosing components is routinely done for all types of parenteral products, the study is usually limited to an examination of the loss of active or an increase in degradates caused by interactions with the

components. For emulsions, it is recommended that a check of the droplet characteristics also be examined during these types of “in-use” studies to ensure that any change in the droplet size distribution does not push the number of large droplets into a range that would pose a risk for patient safety.

Functional Excipients/In-Process Stability

The components of an emulsion are complex products derived from natural sources and thus bring with them inherent variability with respect to their composition. The typical emulsifier, lecithin, is available in a range of purities that have different chemical compositions. The compositions vary in their relative amounts of phosphatidylcholine, phosphatidylethanolamine, and nonpolar lipids. However, the grade of lecithin does not impact the route of degradation which generally proceeds by first-order hydrolysis of the aliphatic chains into free fatty acids. Given that water is always present in large quantities during production and storage of the material, the reaction will occur over time but can occur at different rates depending on the exposure conditions. The change in the ratio of lecithin to free fatty acid can affect the physical stability of the emulsion as free fatty acids do not have the same emulsification properties as lecithin.

The processing conditions can have a dramatic impact on the amount of intact lecithin (typically measured by phosphatidylcholine) that remains in the product and the resulting amount of free fatty acids that are formed. Exposure to pH extremes (<3 and >9) as well as high temperatures will accelerate the hydrolysis of lecithin into the free fatty acid degradation products. Since no process will always operate free of deviations, it is advantageous to understand what process variations can do to the overall product composition.

To help understand the impact of process deviations, the coarse emulsion and fine emulsion should be evaluated separately if possible; however, the final emulsion may be used as a surrogate if needed. During the preparation of the coarse emulsion, the emulsion is typically adjusted to a target pH with base which is a potential source of lecithin degradation. The literature indicates that the degradation of lecithin in strongly alkaline conditions is extremely rapid with nearly 70% being consumed in just 60 minutes (50). Stability studies of very short duration at higher pH values will be valuable in assessing the potential impact that poor mixing during pH addition or excess addition of base would have on the free fatty acid levels, achievable droplet size distribution, and resulting PFAT5 concentrations in the final product.

In the absence of pH extremes, the hydrolysis of the lecithin will still occur according to Arrhenius kinetics over the probable temperature ranges for a product of this type (25–125°C) (51). This degradation is very easy to monitor at accelerated conditions to determine the approximate amount of lecithin degradation that will occur over the shelf-life of the product. Simple stability studies at 60°C or 80°C will be valuable in determining the expected physical characteristics of the emulsion at expiry. These studies should be leveraged to ensure that the formulation is not operating at the edge of a critical lecithin concentration range that will cause physical instability as the lecithin degrades. These studies will also help to support hold times of the emulsion during processing.

CONCLUSION

The assessment of the stability of small molecule parenteral drug products shares many commonalities with the development of solid oral dosage forms. For example, forced stress experiments to evaluate the susceptibility of a drug product to degrade upon storage in the presence of excipients and the impact of light, moisture, and temperature are typically relatively standard practice. Parenteral drug products formulated in liquid form or freeze-dried highlight some of the unique features of these drugs. For sterile liquid formulations for injection, evaluation of the sterilization process, which is often achieved via steam sterilization, is critical. Here not only achieving the desired reduction in Cfu, but also ensuring that no drug or excipient

degradation occurs is critical. Steam autoclaving may cause the color of solution to change, increase viscosity, or lead to particulate formation.

Lyo products add complexity as far as solid-state hydrolysis is concerned. The driving force here might be the moisture available from the rubber closures which require washing and steam autoclaving for sterility prior to use. Stopper drying conditions are essential to maintain to control the amount of water available to the finished product over the shelf-life. These drying conditions have to be efficient in removal of water but also gentle enough to ensure that the container closure integrity is not compromised over the shelf-life of the product. Dosing a sterile injectable also requires careful evaluation of the formulation compatibility with any commercial diluents that may be involved in the dosing process. A detailed understanding of the entire patient administration process is essential to ensure that no degradation occurs with exposure to light, which is typically much more problematic with dilute solutions.

REFERENCES

1. Nail SL. Physical and chemical stability considerations in the development and stress testing of freeze-dried pharmaceuticals. In: Baertschi SW, ed. *Pharmaceutical Stress Testing*. Vol. 153, New York: Taylor & Francis, 2005: 261–91.
2. Fasani E, Albini, A. Photostability Stress testing. In: Baertschi SW, ed. *Drugs and the Pharmaceutical Sciences*. Vol. 153, Taylor & Francis, 2005: 293–319.
3. Antipas AS, Landis, Margaret S. Solid State Excipient Compatibility Testing. In: Baertschi SW, ed. *Drugs and the Pharmaceutical Sciences*. Vol. 153, New York: Taylor & Francis, 2005: 419–58.
4. Clapham D, Templeton, Allen C. Practical aspects of conducting photostability stress testing. In: Baertschi SW, ed. *Drugs and the Pharmaceutical Sciences*. Vol. 153, New York: Taylor & Francis, 2005: TBD.
5. Q1B. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human use. *Stability Testing: Photostability Testing of New Drug Substances and Products Q1B*, 1996.
6. Reed RA, Harmon P, Manas D, et al. The role of excipients and package components in the photostability of liquid formulations. *PDA J Pharm Sci Technol* 2003; 57: 351–68.
7. Q3B(R2). International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human use. *ICH Harmonised Tripartite Guideline: Impurities in New Drug Q3B(R2)*, 2006.
8. Q1A(R2). International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human use. *ICH Harmonised Tripartite Guideline: Stability Testing of New Drug Substances and Products Q1A(R2)*, 2003.
9. Abrahamson HB, Rezvani, AB, Brushmiller G. Photochemical and spectroscopic studies of complexes of iron(III) with citric acid and other carboxylic acids. *Inorg Chim Acta* 1994; 226: 117–27.
10. Faust B, Zepp, RG. Photochemistry of aqueous iron(III)–polycarboxylate complexes: roles in the chemistry of atmospheric and surface waters. *Environ Sci Tech* 1993; 27: 2517–22.
11. Haber FaW, J. Über die Katalyse des Hydroperoxydes. *Naturwissenschaften* 1932.
12. Jennings TA. *Lyophilization: Introduction and Basic Principles*. Vol. 1, Buffalo Grove, IL, USA: Interpharm Press, 1999.
13. USP <789> Particle Matter in Injections.
14. Cernik M. Stability of pharmaceutical products. *CHEMMagazin* 2009; 19: 32–4.
15. Baertschi SW, Alsante, Karen M. Stress testing: relation to the development imeline. In: Baertschi SW, ed. *Drugs and the Pharmaceutical Sciences*. Vol. 153, New York: Taylor & Francis, 2005: 173–9.
16. Nussbaum MA, Jansen, Patrick J, Baertschi, Steven W. Role of “mass balance” in pharmaceutical stress testing. In: Baertschi SW, ed. *Drugs and the Pharmaceutical Sciences*. Vol. 153, New York: Taylor & Francis, 2005: 181–204.
17. Boccardi G, Harmon, Paul A. Oxidative susceptibility testing. In: Baertschi SW, ed. *Drugs and the Pharmaceutical Sciences*. Vol. 153, New York: Taylor & Francis, 2005: 205–34.
18. Carlson E, Chandler W, Galdo I, Kudla T, Ta C. Automated integrated forced degradation and drug-excipient compatibility studies. *JALA* 2005; 10: 374–80.
19. Zoglio MA. Preformulation stability testing of parenteral products. *Bull Parenter Drug Assoc* 1968; 22: 295–8.

20. Degradation pathways of drug molecule A.
21. Wasylaschuk WR, Harmon PA, Wagner G, et al. Evaluation of hydroperoxides in common pharmaceutical excipients. *J Pharm Sci* 2006; 96: 106–16.
22. Pikal MJ, Dellerman K, Roy ML. Formulation and stability of freeze-dried proteins: effects of moisture and oxygen on the stability of freeze-dried formulations of human growth hormone. *Dev Biol Stand* 1991; 74: 21–38.
23. Kristensen S. Photostability of parenteral products. In: H.H. T, ed. *Photostability of Drugs Drug Formulations*, 2nd edn. Oslo: Taylor & Francis, 2004: 303–30.
24. Templeton AC, Bowen WE, Klein LJ, Harmon PA, Lu Y, Reed RA. Unexpected photochemistry in pharmaceutical products: a review on the role of diluents, excipients, and product components in promoting pharmaceutical photochemistry. *Drugs Pharm Sci* 2007; 163: 223–51.
25. Ludwig JD NPD, Davis CW. Automated method for determining Instron residual seal force of glass vial/rubber closure systems. *J Parenter Sci Technol* 1993; 5: 211–53.
26. Burrell LS CMW, DeMuth GE, Lambert WJ. Development of a dye ingress method to assess container-closure integrity: correlation to microbial ingress. *PDA J Pharm Sci Technol* 2000; 54: 449–55.
27. Bergquist PA, Zimmerman J, Kenney RR, Han RY-H, Hunke WA. Stability of tirofiban hydrochloride in three commonly used I.V. Solutions and polyvinyl chloride administration sets. *Am J Health-Syst Pharm* 1999; 56: 1627–9.
28. Moore DE. Photophysical and photochemical aspects of drug stability. In: Tonnesen HH, ed. *Photostability of Drugs and Drug Formulations*. Oslo: Taylor & Francis (London, UK), 1996: 9–38.
29. Templeton AC, Xu H, Placek J, Reed RA. Implications of photostability on the manufacturing, packaging, storage, and testing. *Drugs Pharm Sci* 2005: 68–86.
30. Bergquist PA, Manas D, Hunke WA, Reed RA. Stability and compatibility of tirofiban hydrochloride during simulated Y-site administration with other drugs. *Am J Health-Syst Pharm* 2001; 58: 1218–23.
31. Corveleyn S, De Smedt S, Remon JP. Moisture absorption and desorption of different rubber lyophilisation closures. *Int J Pharm.* 1997; 159: 57–65.
32. Pikal MJ, Shah S. Moisture transfer from stopper to product and resulting stability implications. *develop Biol Standard* 1991; 74: 165–79.
33. Vromans H, van Laarhoven JAH. A study on water permeation through rubber closures of injection vials. *Int J Pharm.* 1992; 79: 301–8.
34. VanAmerogan GJ. Diffusion in elastomers. *Rubber Chem Technol* 1964; 37: 1065–152.
35. Solomun L, Ibric S, Boltic Z, Djuric Z, Stupar B. The impact of primary packaging on the quality of parenteral products. *J Pharm Biomed Anal* 2008; 48: 744–8.
36. Abend A. RRA, Templeton A, C. Impact of stopper dryness for lyophilized drug products. *Am Pharm Rev* 2006; 6: 35–8.
37. Buck AL. New equations for computing vapor pressure and enhancement factor. *J Appl Meterol* 1981; 20: 1529.
38. Perry RHaG, D.W. Perry's Chemical Engineer's Handbook. In: Perry RHaG, DW, ed., New York: McGraw-Hill, 2007.
39. Templeton AC, Placek J, Xu H, Mahajan R, Hunke WA, Reed RA. Determination of the moisture content of bromobutyl rubber stoppers as a function of processing: implications for the stability of lyophilized products. *PDA J Pharm Sci Technol* 2003; 57: 75.
40. Wang YJ, Dahl TC, Leesman GD, Monkhouse DC. Optimization of autoclave cycles and selection of formulation for parenteral products: part II. Effect of counter-ion on pH and stability of diatrizoic acid at autoclave temperatures. *J Parenter Sci Technol* 1984; 38: 72–7.
41. Held HR, Landi S. Water permeability of elastomers. *J Biol Stand* 1977; 5: 111–19.
42. Carlisle CB, Cooper DE. Tunable diode laser frequency modulation spectroscopy through an optical fiber: high-sensitivity detection of water vapor. *Appl Phys Lett* 1990; 56: 805–7.
43. Carstensen JT. Effect of moisture on the stability of solid dosage forms. *Drug Dev Ind Pharm* 1988; 14: 1927–69.
44. Zhang X, Kirsch LE. An assessment of techniques for evaluating the physical stability of parenteral emulsions. *PDA J Pharm Sci Technol* 2003; 57: 300–15.
45. Li LC, Parasrampuria J, Bommireddi A, Pec E, Dudleston A, Mayoral J. Moist-heat sterilization and the chemical stability of heat-labile parenteral solutions. *Drug Dev Ind Pharm* 1998; 24: 89–93.
46. Masson G, Trottet B. Physical stability of sterilized oil-in-water emulsions intended for total parenteral nutrition. *Rev Fr Corps Gras* 1984; 31: 391–4.

47. Canada T. *Nutr Clin Pract* 2006; 21: 636–7.
48. Driscoll DF. Formulation of parenteral and enteral admixtures. *Update Intensive Care Emerg Med* 2000; 34: 138–50.
49. Driscoll DF, Ling, Pai-Ra, Bistran, Bruce R. *J Parenter Enteral Nutr*, 2007; 31: 148–53.
50. Dennis Ka. *Biochemistry* 1981; 20: 6079–85.
51. Herman and Groves. *J Pharm Pharmacol* 1992; 44: 539–42.

13 | Stability considerations in development of freeze-dried pharmaceuticals

Steven L. Nail

INTRODUCTION

Critical quality attributes of freeze-dried injectable pharmaceuticals include sterility, freedom from pyrogens, and freedom from extraneous particulate matter. These attributes are achieved by appropriate processing conditions. In addition, these products must completely recover their original activity when reconstituted with water, should be quickly and easily reconstituted, and should retain these attributes over the shelf-life of the product. Stability assessment and shelf-life prediction is usually a major focus of a pharmaceutical scientist's attention in the development of freeze-dried dosage forms, and stability studies have a critical impact on the time course of product development. Stability of drugs as freeze-dried solids is a concern for both small molecules and large molecule drugs, although there are important differences between these two arenas. For stability assessment of freeze-dried small molecules, the main concern is the influence of the physical state of a drug on stability, where the amorphous form may in some cases be at least an order of magnitude less stable than the same drug in its crystalline state (36,51). Acute damage by the freeze-dry process itself is hardly ever a concern. For freeze drying of large molecules, where proteins are the main focus of this discussion, there are two types of stability—short-term stability against loss of protein integrity caused by stresses associated with freezing and freeze drying, and long-term stability, referring to the potential for loss of integrity during storage of the freeze-dried solid (3). Because of the structural complexity of proteins and the vital role of water in determination of secondary and tertiary structure, freeze drying can, in some cases, result in complete loss of activity. The role of water in maintenance of protein structure may account for another important difference between small molecules and proteins in the context of freeze drying. For small molecules, the conventional wisdom that “dryer is better” in terms of storage stability nearly always applies. For proteins, this is not always the case, since the integrity of some proteins after freeze drying can be adversely affected by over drying.

The ability to select formulation and processing conditions that would result in maximum stability prior to running real-time stability studies would accelerate pharmaceutical product development enormously. Of course, this would require detailed understanding of degradation mechanisms and the type of molecular mobility that is relevant to these degradation mechanisms. Armed with this knowledge, and the appropriate means to measure the relevant molecular mobility, it would be possible to screen formulations for optimum stability characteristics. While we have not reached this degree of sophistication yet, significant advances have been made in recent years for both small and large molecules.

The purpose of this chapter is to present a brief overview of the physical chemistry of freezing and freeze drying, and to discuss the influence of both formulation and processing considerations on stability of the finished dosage form for both small and large molecules. Investigations into measurement of molecular mobility and its relevance to solid-state stability are reviewed. The scope of the discussion is limited to freeze drying from aqueous solutions only, and does not include freeze drying from cosolvent systems or freeze drying of dispersed systems such as suspensions or emulsions.

A BRIEF OVERVIEW OF THE FREEZE-DRYING PROCESS

Freeze drying is used to remove water from heat-sensitive substances at low temperature by the process of *sublimation*, where water is removed via a phase change from a solid to a vapor without passing through a liquid state. This takes place below the triple point of water, at approximately 0°C and 4.5 mm of mercury (Hg). The main advantage of freeze drying over

other drying unit operations is that water is removed at very low temperatures, thus avoiding the thermal damage to heat-sensitive materials that is associated with other drying operations. Another advantage is that, properly done, freeze-dried solids have a relatively high specific surface area, and this facilitates rapid, complete reconstitution. Given that freeze-drying avoids many of the problems associated with chemical and physical stability of drugs in aqueous solution, development of a freeze-dried product usually has a higher probability of technical success than development of an aqueous solution formulation, and this can be an advantage when considering the time required for pharmaceutical development.

Freeze drying is not without limitations, however. A significant limitation can be stability of the drug as a freeze-dried solid. The other is cost, both in terms of capital investment and operation. Pharmaceutical freeze dryers are expensive because of the very large size of modern freeze dryers, materials of construction, the number of subsystems involved (refrigeration, vacuum pumps, clean-in-place (CIP), sterilize-in-place (SIP), process monitoring and control, to name a few) and the amount of redundancy needed in light of the high value of the products being processed. In terms of operating cost, drying operations are the most expensive unit operations common to pharmaceutical processing, largely because of the high heat of fusion of water, and freeze drying is the most expensive of the drying operations. One of the main reasons for this is that heat transfer in freeze drying is very inefficient because the process takes place under vacuum. More traditional drying operations might take only a few hours. Freeze drying, on the other hand, takes days—perhaps three, four, or more. It behooves pharmaceutical scientists, therefore, to optimize freeze-dry process conditions; that is, develop process conditions that minimize overall processing time while maintaining the quality attributes of the product.

A cross-sectional schematic of a pharmaceutical freeze dryer is shown in Figure 1. Operationally, freeze drying of a final dosage form usually consists of filling glass vials with an aqueous solution of the solutes to be freeze dried, partially inserting a special slotted rubber stopper

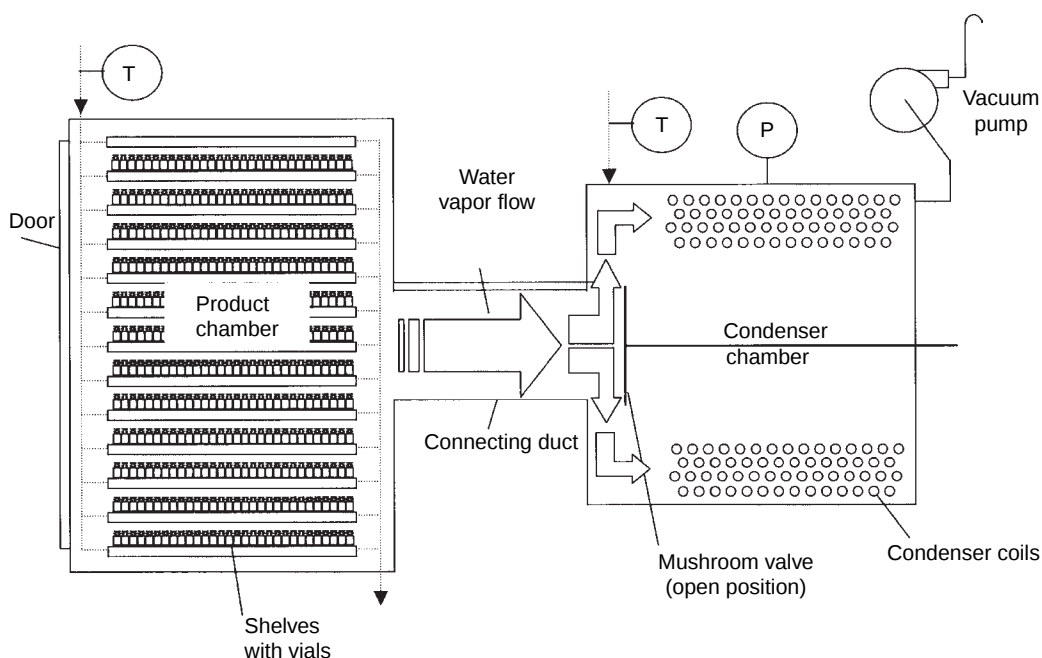


Figure 1 Schematic diagram of a typical pharmaceutical freeze dryer. Drawing provided by Dr. Jim Searles, Activ-Dry, Inc.

that allows water vapor to flow through slots in the stopper when in the partially inserted position, and transferring the vials to the shelves of the freeze dryer. Temperature sensors are often placed in a few vials to monitor product temperature. The shelves are then cooled to a temperature in the range of -40°C to -50°C . The vials are held long enough to approach thermal equilibrium with the shelves, typically for a minimum of 3 to 4 hours. Pressure in the freeze dryer is then reduced to a level less than the vapor pressure of ice at the temperature of the product. For example, for a product temperature of -40°C , the pressure in the system would be reduced to less than 0.096 mm Hg (96 μ Hg). In order for sublimation to take place, energy must be provided in a quantity equal to the heat of sublimation of ice, ΔH_s , which is approximately 2800 J/gm. This is accomplished by heating the transfer fluid and warming the shelves to a temperature high enough to effect sublimation, but not as high as to melt the frozen material in contact with the bottom of the vial or to collapse the partially dried product. The partial pressure of water vapor in the chamber is maintained at a low level by the condenser, which typically operates at temperatures between -65°C and -80°C , and water vapor is removed from the product primarily by a process of *bulk flow* from a region of relatively high pressure (the sublimation front) to low pressure (the condenser). This phase of the process, called *primary drying*, is characterized by a visible receding boundary from the top of the frozen layer. When the ice has sublimed, the heat of sublimation is no longer needed, and the product temperature usually increases sharply toward the shelf temperature.

In general, not all of the water initially present in the product is converted to ice during freezing. The quantity of unfrozen water is a function primarily of the composition of the formulation and, to a lesser extent, the thermal history of the freezing process. This is discussed in more detail below. Removal of this “unfrozen” water, which may be 20% or more of the weight of dry solids, is called *secondary drying*. During secondary drying, the shelf temperature is usually increased, since ice is no longer present. In contrast to primary drying, where water vapor is removed by bulk flow, water vapor removal during secondary drying is largely by a process of diffusion, or flow by molecular motion from a region of high concentration to a region of lower concentration. At the end of secondary drying, the shelf stack is compressed together, causing insertion of the lyostoppers to the fully stoppered position. This may be done under full vacuum, partial vacuum, or at atmospheric pressure. For drugs that are sensitive to oxidation in the solid state, the composition of gas in the headspace of the vial should be considered part of the formulation. An example is shown in Figure 2 for propantheline bromide, a compound subject to oxidation in the solid state. Formation of the 9-hydroxy degradation product is rapid if vials are stoppered under partial atmospheric pressure after venting the freeze dryer

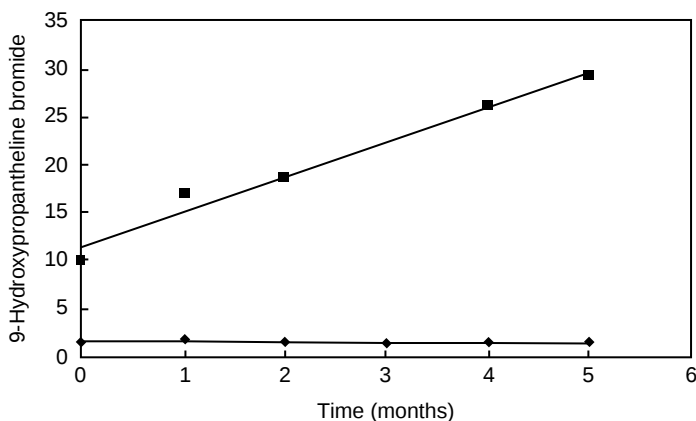


Figure 2 Oxidation of propantheline bromide in the solid state during storage at 40°C following stoppering under air (closed square) or nitrogen (closed diamond).

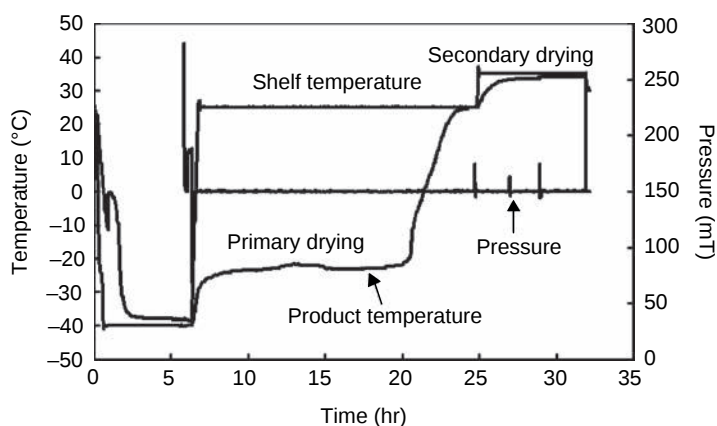


Figure 3 Process variables for freeze drying, including shelf temperature, chamber pressure, and product temperature. As primary drying is completed, the product temperature increases rapidly, as ice is no longer present.

with air, whereas the product is stable if the freeze dryer is vented with nitrogen. In cases like this, it is important to monitor the headspace composition during stability testing, either by measuring oxygen levels in the headspace or by measuring pressure in the vial in the event that there is a breach in container/closure integrity. Commercial instrumentation is now available for nondestructive monitoring of the vial headspace for oxygen level, moisture, and total pressure using frequency modulation spectroscopy (FMS).

Since the driving force for freeze drying is the vapor pressure of ice, it is important from the standpoint of process efficiency to keep the product temperature as high as practical during primary drying. However, the product temperature must be maintained below the maximum allowable product temperature, which is either a eutectic melting temperature or a collapse temperature (see discussion below). Monitoring of product temperature is therefore important for establishing optimum process conditions during product development. A typical plot of process variables is shown in Figure 3.

THE PHYSICAL CHEMISTRY OF FREEZING AND ITS RELEVANCE TO FREEZE DRYING

An understanding of the physical chemistry of freezing is essential to understanding how both formulation composition and process conditions influence the quality of a freeze-dried product, including stability. A schematic diagram of the physical events occurring during freezing is shown in Figure 4. Supercooling, or retention of the liquid state below the equilibrium freezing point of the solution, always occurs to some extent—it is not uncommon for aqueous solutions to supercool by 12°C or more. The effect of supercooling is to limit the ability to control the freezing rate by manipulation of shelf temperature, since the greater the degree of supercooling, the faster the effective rate of freezing once ice crystals nucleate.

As indicated in Figure 4, ice may not be the first component of the solution to crystallize. As the temperature is decreased, the equilibrium solubility of one or more solutes may be exceeded, allowing nucleation and crystal growth. An example of this behavior is illustrated by the drug pentamidine isethionate where, depending on the thermal history of freezing, either drug or ice may nucleate first (14). Process validation studies should include determination of whether the thermal history of freezing (for example, placing vials on precooled shelves versus loading with the shelves at room temperature and slowly decreasing the shelf temperature during freezing) has a measureable impact on product quality, such as activity, residual moisture, appearance of solids, reconstitution time, physical state of solids, and stability characteristics.

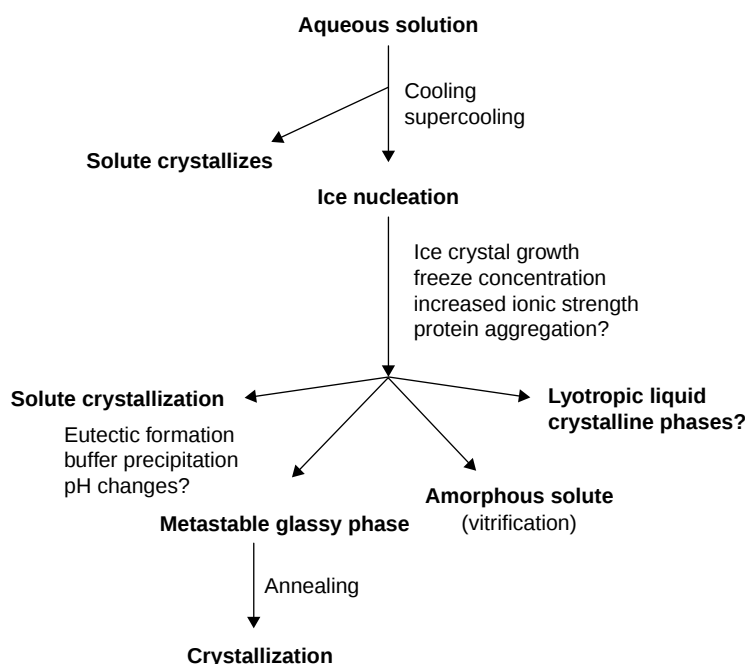


Figure 4 Schematic of physical events taking place during freezing of an aqueous solution.

As ice crystals grow in the freezing system, the solutes are concentrated. Increased ionic strength accompanying the freezing process is often an important consideration in freeze drying of proteins as well as other biological materials, since high ionic strength may promote denaturation of proteins as well as osmotic dehydration of microorganisms. In addition, rates of some chemical reactions—particularly second-order reactions—may be accelerated by freezing through this freeze-concentration effect. Examples include reduction of potassium ferricyanide by potassium cyanide (21), oxidation of ascorbic acid (22), and polypeptide synthesis (42). Kinetics of reactions in frozen systems has been reviewed by Pincock and Kiovsky (39). These effects are generally not significant over the time course of freezing as a part of the freeze-dry process. They can be significant, however, in the context of stability during storage in the frozen state, and storage of intermediate materials during bioprocessing is commonly done.

A key consideration in the physical chemistry of freezing is the fate of solutes as a result of the freezing process; that is, the solute(s) may either crystallize from a freezing system, or a glassy mixture may be formed. The fate of the solute in the freeze-concentrated solution is a key concept in understanding the material science of freeze drying.

SOLUTE CRYSTALLIZATION DURING FREEZING—EUTECTIC MIXTURE FORMATION

For the sake of simplicity, we will consider freezing of a solution containing only one solute. As indicated in Figure 4, there are several possibilities, the simplest of which is crystallization of solute from freeze-concentrated solution to form a simple eutectic mixture with ice. A eutectic mixture is an intimate physical mixture of two or more crystalline solids that melts as if it were a single, pure compound. The relevance of the eutectic temperature to freeze drying is that it represents the maximum allowable product temperature during primary drying, since eutectic melting would introduce liquid water and destroy the desirable properties of a freeze-dried solid. Melting of the frozen system during primary drying is sometimes called *meltback*. A photomicrograph of a frozen solution of sodium chloride, and the corresponding eutectic melt at about -21.5°C , is shown in Figure 5.

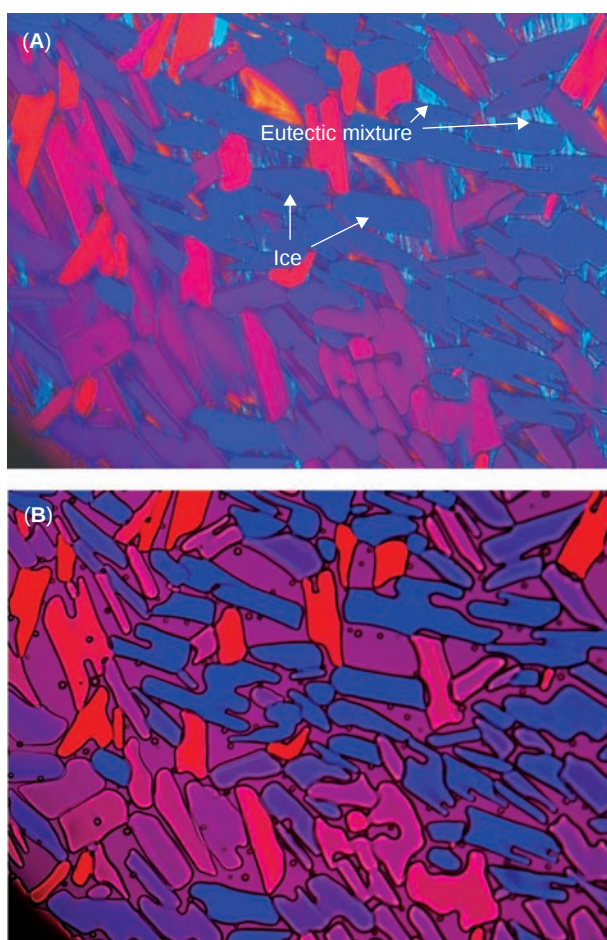


Figure 5 Photomicrograph of a frozen solution of 10% sodium chloride illustrating the eutectic mixture (A) and the melted eutectic mixture (B). For sodium chloride/ice, the eutectic melt occurs at about -21.5°C .

Crystallization of phosphate buffers during freezing is a special case of eutectic mixture formation during freezing, and is worthy of mention not only because phosphate salts are the most common buffers used in freeze-dried pharmaceutical formulations, but also because the crystallization process can cause significant shifts in pH during freezing. This arises because conjugate acid and bases in equilibrium do not have the same aqueous solubility. For the sodium phosphate buffer system, the dibasic salt tends to precipitate, and this causes a decrease in the pH of the system (46,47,18), and pH shifts of up to three units have been reported (37). It is important to remember that, in order for a pH shift to be observed, one of the buffer species must crystallize—the mere presence of a phosphate buffer in a formulation does not mean that the pH will shift during freezing. Gomez et al. reported that solutes such as sucrose and mannitol inhibited crystallization of buffer species, resulting in smaller pH shifts upon freezing. The presence of sucrose and mannitol at concentrations above 3 moles per mole of dibasic sodium phosphate completely prevented salt crystallization. As a general formulation guideline, though, buffer concentration should be kept to a minimum (58).

Other pharmaceutically relevant buffer systems have not been as well characterized as phosphate with respect to pH changes accompanying freezing. Larsen (31) reported that

acetate, citrate, glycine, and Tris show only small pH shifts upon freezing. Sundarmurthy and coworkers (45) have investigated pH “swings” in sodium succinate buffer systems (pK_a 4.2, 5.6) during freezing. When buffered to a pH <5.6, the pH of the freeze concentrate first increased, and then decreased due to sequential crystallization of succinic acid, monosodium succinate, disodium succinate. When buffered to pH > 5.6, the pH of the freeze concentrate first decreased, and then increased due to sequential crystallization of disodium succinate, monosodium succinate, succinic acid. The pharmaceutical significance of such pH swings remains to be explored, but such observations do underscore the point that pH changes in freeze-concentrated systems can be much larger than they would be if a buffer were simply excluded from the formulation.

There are no published studies which examine the significance of pH shifts on quality attributes of freeze-dried formulations of small molecules. However, Pikal-Clelland and coworkers (37), using monomeric and tetrameric β -galactosidase as model systems, reported lower recovery of activity when freeze/thawing from a solution containing sodium phosphate buffer (where up to a three pH unit decrease was observed) than from a potassium phosphate buffer (a 0.3 pH unit increase). Secondary structural perturbations as measured by FTIR spectroscopy were also greater in the sodium phosphate buffer system. When these systems were freeze dried and reconstituted, tetrameric β -galactosidase showed significantly more loss of activity when freeze dried from the sodium phosphate buffer system relative to the potassium phosphate buffer.

POLYMORPHISM AND HYDRATE FORMATION ACCOMPANYING FREEZING

While not a thoroughly studied aspect of freezing of pharmaceutical formulations, solutes that crystallize from freezing solutions may form different polymorphs during freezing under different conditions, arising either from different thermal histories of freezing, or freezing from solutions of different composition. Mannitol, a very common bulking agent in both small-molecule and large-molecule formulations, can crystallize as one of three different anhydrate crystal forms (8,30) or as a hydrate, first reported by Yu and coworkers (57), and later characterized by Nunes et al. (34) as a hemihydrate. Yu and coworkers reported that the hydrate is metastable, readily converting to the anhydrate, and that considerable vial-to-vial variability in the relative amount of hydrate was observed. Hawe and Friess (23) reported that freeze drying at product temperatures below about -30°C promotes the formation of mannitol hydrate, and that annealing also promotes mannitol hydrate formation. Hydrate formation may be important in solid-state stability, both because of the potential for release of the water for hydration during storage and because of the potential for vial-to-vial variability in the effective level of residual moisture. While the distribution of crystal forms of anhydrous mannitol varies with both formulation composition and processing conditions, there appear to be no reports of anhydrous mannitol crystal forms having a measurable influence on quality attributes of the freeze-dried product, such as reconstitution time or solid-state stability.

Because of the tendency of mannitol to crystallize from freezing aqueous systems, it would be a poor choice as a stabilizer for a protein since, in order for it to be effective as a stabilizer, a protective solute must remain amorphous. Mannitol is often used in combination with a disaccharide, where the disaccharide serves as the protective solute, and mannitol, being crystalline, acts as a physical stabilizer for the cake, thus inhibiting macroscopic collapse of the cake. This stabilization can allow for freeze drying under more aggressive processing conditions than when a disaccharide alone is used.

Glycine is another common excipient in freeze-dried formulations, particularly for proteins. Glycine has been reported to crystallize into three different polymorphs, depending on formulation and the thermal history of freezing (2,13). There are unpublished reports of the distribution of glycine polymorphs having a measurable impact on reconstitution time, where the presence of significant amounts of the gamma crystal form may be associated with prolonged reconstitution.

Of course, the drug can crystallize in different ways, depending on formulation and processing conditions, although there are few published reports of this. Pentamidine isethionate is an example (14).

SOLUTE REMAINS AMORPHOUS DURING FREEZING—GLASSY MIXTURE FORMATION

Many solutes do not crystallize from a freeze-concentrated aqueous solution. Instead, a *glassy mixture* of water and solute is formed in the interstitial space between ice crystals. The *glass transition temperature*, a reversible change in state between a mechanically solid material and a viscous liquid, is an important property of this glassy mixture. This glass transition temperature provides the physical-chemical basis for *collapse* in freeze drying. During freeze drying, ice crystals are removed by sublimation. If freeze drying takes place below the glass transition temperature of a frozen system containing a solute that remains amorphous, the material left behind after ice removal is mechanically rigid, and the microstructure that was established by freezing is retained in the freeze-dried solid. If, on the other hand, freeze-drying takes place above the glass transition temperature, then the solids left behind may not be rigid enough to support their own weight. The viscous flow of the partly dried solids results in collapse. The resulting product is not only pharmaceutically inelegant, but may also reconstitute slowly, and may contain a relatively high level of residual moisture, which can adversely affect stability.

DIFFERENCES IN FREEZE-DRYING BEHAVIOR BETWEEN AMORPHOUS AND CRYSTALLINE SOLUTES

If the solute crystallizes completely during freezing, the microstructure of the system is illustrated by the sketch in Figure 6, where the interstitial material between the ice crystals consists of an intimate mixture of small crystals of ice and solute. For an amorphous solute, the interstitial material consists of a glassy mixture of solute and unfrozen water. For a eutectic-forming system, the upper product temperature limit during primary drying is the eutectic melting temperature, whereas for an amorphous system the upper temperature limit is the collapse temperature. While eutectic melting temperatures of ice/inorganic salt mixtures can be quite low (the eutectic melting temperature of calcium chloride/water is about -52°C), eutectic melting temperatures of ice and most organic compounds are in the range of just below 0°C to perhaps -15°C . These relatively high melting temperatures allow for maintenance of high product temperatures during primary drying, resulting in a more efficient drying process. Collapse temperatures are generally much lower than eutectic melting temperatures. Collapse temperatures below -40°C are not uncommon, particularly for protein formulations containing sugars as protective solutes in combination with added salts. Small molecules that do not

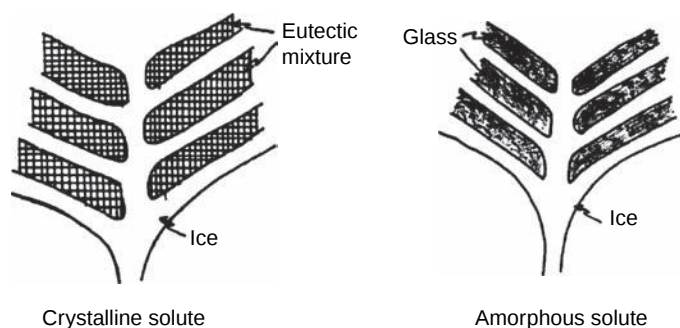


Figure 6 For solutes that crystallize during freezing, the interstitial space between ice crystals consists of an intimate physical mixture of crystalline solute and ice (a eutectic mixture). For solutes that remain amorphous, the interstitial space is a glassy mixture of solute and unfrozen water.

crystallize from a freezing aqueous system can also have low collapse temperatures. For example, when glycine remains amorphous, it collapses below -60°C (when glycine crystallizes, the melting point of the ice/glycine eutectic mixture is about -3.5°C). Given that most production-scale freeze dryers are unable to consistently maintain product temperatures below about -40°C , freeze drying is not feasible for all formulations. Even if the product temperature could be controlled at temperatures below -40°C , the vapor pressure of ice at such low temperatures; therefore the driving force for freeze drying, is relatively low. Since the vapor pressure of ice at -40°C is only 0.096 Torr, the system pressure must be kept well below this pressure, which can be a significant demand on the vacuum system. As a general formulation guideline, formulations with collapse temperatures of -40°C or lower should be avoided.

As illustrated in Figure 6, nearly all water is present as ice (either preeutectic or eutectic) when the solute crystallizes. As the sublimation front moves through the frozen system, essentially all of the ice can be removed by a process of *bulk flow*, or transport of water vapor from a region of high pressure to lower pressure, through the porous bed of dried solid. There is essentially no secondary drying. For the sucrose/water system, representing an amorphous solute, there is about 20% water associated with sucrose, which must be removed during secondary drying. This water must be removed by a process of *diffusion*, or flow by molecular motion from a region high concentration to a region of lower concentration. This combination of a significant amount of water to be removed by secondary drying and the slower transport mechanism often means that secondary drying is a significant part of the total drying time needed.

Of course, it is common (and often desirable) to have both amorphous and crystalline phases present in a freeze-dried formulation. This is particularly relevant to freeze-dried proteins, where the lyoprotectant is present in the amorphous state, and another component, such as glycine or mannitol, is present as a crystalline solid in order to impart mechanical integrity and pharmaceutical elegance to the lyophilized solid. Such systems may exhibit *microcollapse*, where the amorphous component of the formulation collapses around a supporting structure of crystals, such that there is no collapse that is visually observable in the freeze-dried solid. Microcollapse is not necessarily a problem as long as quality attributes of the final product are maintained.

METASTABLE GLASS FORMATION

In addition to eutectic crystallization and glass formation (vitrification), there are other types of freezing behavior, which may be observed (Fig. 3). A metastable glass may form which, with subsequent heating, undergoes crystallization. The most common example of metastable glass formation is mannitol, which crystallizes when heated above its T_g' (see discussion of thermal analysis below). This type of behavior is the basis for *annealing* during freeze drying (see in the following), which refers to warming the product after an initial freezing step, generally to a temperature above T_g' but below the onset of melting, holding for a period of perhaps 1–4 hours, then cooling the material again before starting drying.

Mannitol is one of the most commonly used excipients in freeze-dried formulations; however, the formulation scientist should be aware of problems associated with the use of mannitol. It is well known that mannitol is associated with vial breakage during freeze drying. This appears to be related to crystallization of mannitol from the metastable amorphous state. Vial breakage associated with mannitol can be minimized by keeping the depth of fill in a vial to no more than about 30% of the overflow capacity of the vial. The thermal history of freezing and the heel radius of the vial (the radius of curvature between the sidewall and the bottom of the vial) may also be significant factors affecting the rate of vial breakage.

MATERIALS CHARACTERIZATION IN FREEZE DRYING

Minimizing empiricism in formulation and process development for freeze-dried products requires characterization of the formulation intended to be freeze dried. The result of such characterization should be information on the upper product temperature limit during the

primary drying stage of freeze drying, knowledge of the physical state of the solute(s), and an assessment of the degree to which the characteristics of the frozen system are affected by changes in composition of the formulation.

In addition to characterizing frozen systems intended to be freeze dried, it is important to characterize the freeze-dried product. This includes determination of the physical state of the dried product, that is, crystalline, partially crystalline, or amorphous. It may also include identification of the crystal form of a component that exhibits polymorphism and determination of whether the crystal form observed is affected by changes in formulation and processing conditions. For amorphous systems, the glass transition temperature of the amorphous solid, as well as the extent to which T_g changes with residual moisture, may be a critical attribute of the product with regard to both physical and chemical stability.

THERMAL ANALYSIS

Thermal transitions commonly observed in frozen systems are illustrated in Figure 7 where, for the sake of this discussion, a deflection upward indicates an endothermic transition. The glass transition is a shift in the baseline toward higher heat capacity. Crystallization during the DSC experiment is observed as an exothermic event, and eutectic melting is an endothermic transition which precedes melting of ice.

Since aqueous systems are prone to supercooling, the most common practice is to record the thermogram during the heating cycle following freezing of the sample. However, the cooling rate of an aqueous solution can have a significant influence on the thermogram recorded during subsequent heating of the sample, particularly for solutes that tend to crystallize during the time course of freezing. The term "critical cooling rate" usually refers to a cooling rate above which crystallization of solute during freezing is prevented. For example, at cooling rates less than about 2°C/min, crystallization of mannitol from a freezing solution takes place during the cooling cycle whereas, at faster cooling rates, crystallization is inhibited, and a metastable glass is formed. In this case, complex thermal behavior is observed in the subsequent heating thermogram, which consists of a glass transition, followed by a melting endotherm and an exotherm indicating crystallization of the solute (25).

For thermal analysis of frozen systems, it is important to remember that the time scales of the thermal analysis experiment and the freeze-drying process are different by orders of magnitude. This is particularly important with respect to solute crystallization, since solutes that do not crystallize appreciably during thermal analysis may crystallize during freezing and freeze drying. Isothermal calorimetry using the DSC, that is, holding a constant temperature and monitoring heat flow, can be a useful method for monitoring the rate of solute crystallization over a longer time than conventional scanning calorimetry.

Thermal analysis is also useful for characterization of the freeze-dried solids. A crystalline solid is usually indicated by a melting endotherm whereas, for an amorphous solid, a glass transition may be observed. An example of a glass transition in an amorphous solid is illustrated in Figure 8 for sodium ethacrylate. For amorphous systems, the glass transition temperature can be an important indicator of physical stability of the formulation. Examination of how the glass transition temperature is influenced by residual moisture can provide useful information toward the establishment of a residual moisture specification for the product. The glass transition temperature of the freeze-dried solid may, or may not, be a useful indicator of chemical stability in the freeze-dried solid state, depending on the type of molecular motion that is relevant to stability.

Thermal analysis can help in development of secondary drying conditions; in particular, the shelf temperature ramp rate during the transition from primary drying to secondary drying and the shelf temperature during secondary drying. For amorphous systems, as unfrozen water is removed from the solid, the system becomes deplasticized and the glass transition temperature of the solid increases. If the rate of increase in product temperature exceeds the

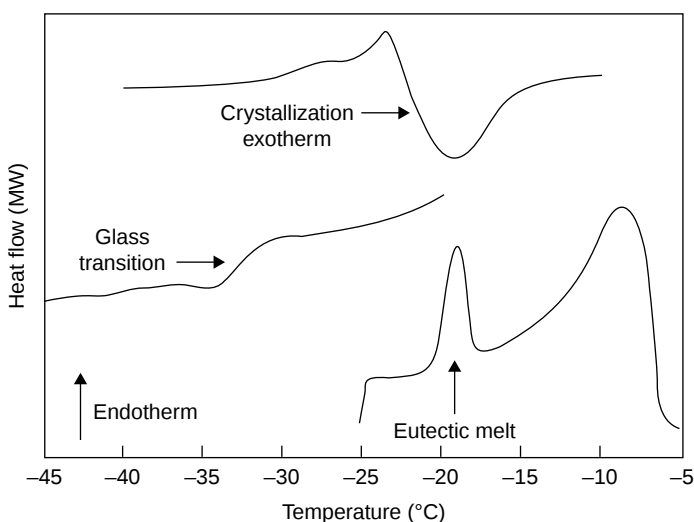


Figure 7 Thermal transitions commonly observed in frozen systems are eutectic melting, glass transitions (T_g), crystallization of solutes, and melting of ice.

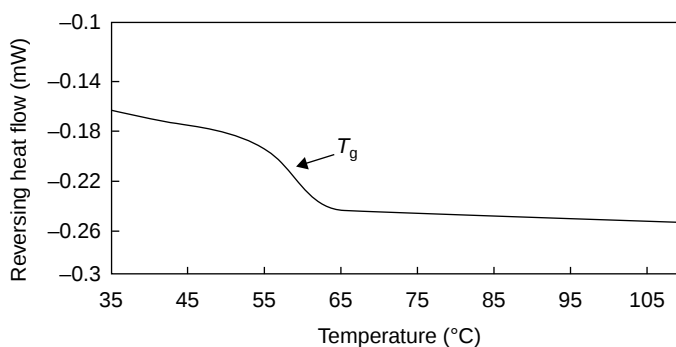


Figure 8 The glass transition temperature of an amorphous freeze-dried solid, shown here for sodium ethacrylate, can be a useful indicator of physical stability of the product.

rate at which the glass transition temperature increases due to deplasticization, then viscous flow can occur during secondary drying, with shrinkage of the cake being the first indication of the onset of collapse.

OPTICAL MICROSCOPY

Optical microscopy using a special low-temperature stage, which can be evacuated to operate at pressures representative of freeze-drying allows for direct observation of materials during freezing and freeze drying, generally under a low magnification of 50–100 $\times$. Experimentally, freeze-drying microscopy is usually carried out by placing a small quantity, perhaps 5 μ L, of sample on a cover slip, freezing the sample, evacuating the system, then systematically varying the sample temperature and observing the resulting morphology in the dried material. This technique is particularly useful for measuring the *collapse* temperature in freeze drying, which may be different from glass transition temperatures measured by thermal analysis. Glass

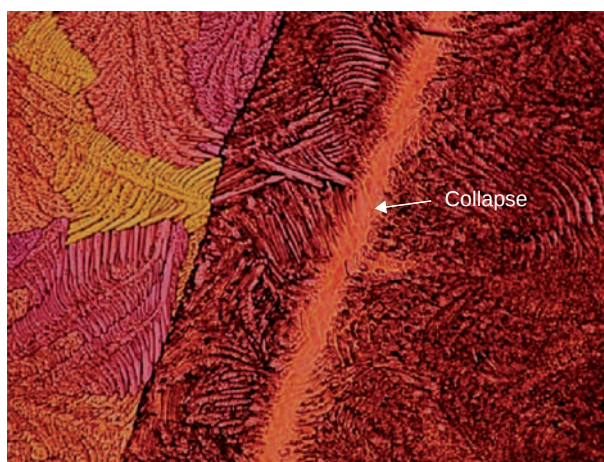


Figure 9 Photomicrograph showing collapse during primary drying.

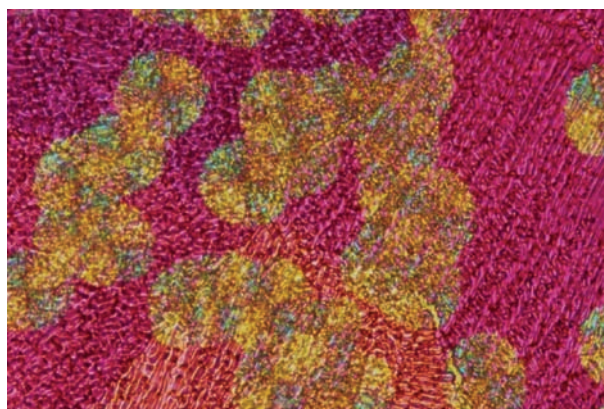


Figure 10 Photomicrograph showing crystallization of a solute (nafcillin) during annealing at -5°C .

transitions are measured on closed systems, whereas collapse is a dynamic phenomenon taking place during freeze drying. It is common for collapse to take place perhaps $2\text{--}3^{\circ}\text{C}$ above T_g' . An example of collapse phenomena is shown in the photomicrograph in Figure 9.

Another application of freeze-drying microscopy is for observation of solute crystallization from frozen systems. The crystallization of nafcillin sodium during annealing at -5°C is shown in Figure 10. The ability to directly observe crystallization helps to establish the necessary time and temperature to facilitate crystallization of a solute during annealing.

STABILITY ASSESSMENT OF FREEZE-DRIED SMALL MOLECULES—THE ROLE OF PHYSICAL STATE OF THE DRUG

The most important factor affecting the chemical and physical stability of a drug as a freeze-dried solid is the physical state of the drug, that is, whether the drug is crystalline, amorphous, or a mixture of crystalline and amorphous forms. Amorphous solids are characterized by a glass transition temperature, T_g' , which represents a reversible change in state from a solid

below T_g to a fluid which exhibits viscous flow over the time frame of interest above T_g . For stability assessment, the time frame of interest is the duration of the stability study, whether at accelerated conditions or at the anticipated storage temperature. Viscous flow during storage can, for example, result in cake shrinkage or even collapse, with subsequent loss of pharmaceutical elegance and potential increased reconstitution time. Storage at temperatures well below T_g is generally adequate to assure physical stability of the product, but does not assure adequate chemical stability, since significant relevant molecular mobility may exist below T_g . As a result of its higher internal energy, the amorphous state has enhanced thermodynamic properties relative to the crystalline state, for example, solubility, vapor pressure, dissolution rate, and chemical reactivity. For this reason, the amorphous state may spontaneously crystallize at temperatures above T_g .

Before proceeding further, it is appropriate to discuss molecular mobility of amorphous solids in the context of long term stability. The temperature dependence of molecular motion in amorphous systems is described by the empirical Vogel–Tammann–Fulcher (VTF) equation:

$$\tau = \tau_0 \exp(B/T - T_0)$$

where τ is the molecular relaxation time, T is the absolute temperature, τ_0 and B are constants, and T_0 is the temperature at which the configurational entropy of the system reaches zero. T_0 is also known as the Kauzmann temperature, and is believed to be roughly 50°C below the experimentally measured glass transition temperature, depending on the nature of the system (see discussion below). Note that, when T_0 is zero, the VTF equation reduces to the Arrhenius equation, where B is analogous to the activation energy.

Glasses have been classified as “strong” or “fragile” depending on the temperature dependence of the activation energy for molecular motion in the region of T_g (19,20). Strong glasses exhibit an Arrhenius temperature dependence of molecular mobility, have small changes in heat capacity at T_g , and broad glass transition regions (when they can be detected at all). Proteins are good examples of strong glass formers. Fragile glasses, on the other hand, undergo much larger changes in heat capacity at T_g and have narrower T_g ranges. The molecular mobility around T_g of a fragile glass is much more temperature dependent than that described by the Arrhenius equation—the molecular mobility may change by an order of magnitude with a 10°K change in temperature, as opposed to roughly a factor of 2 over the same temperature range for mobility that is described by the Arrhenius relationship. The values of B and T_0 in the VTF equation are related to glass fragility. Fragile glasses are characterized by low values of B (<10), and $(T_g - T_0)$ is generally less than 50. Strong glasses, on the other hand, have high values of B (>100), and $(T_g - T_0)$ is usually greater than 50. Another method for estimating the fragility of a glassy system is simply the ratio of the melting temperature to the glass transition temperature (T_m/T_g , in K), where strong glasses have ratios greater than 1.5 (19).

Dramatic differences in stability between crystalline drugs and the same drug in its amorphous form was demonstrated by Pikal and coworkers (36) for potassium penicillin G, cephamandole nafate, and cephamandole sodium, where the rate of degradation of the amorphous form was at least one order of magnitude greater than that of the corresponding crystalline form, even when the amorphous form contained less than 0.1% residual moisture. This study also demonstrated large differences in stability between amorphous forms with different moisture levels. Arrhenius plots for amorphous solids were nonlinear, with activation energy decreasing with increasing temperature. Based on the discussion above, a nonlinear Arrhenius plot is not surprising. This has important implications for accelerated stability testing, since incorrect predictions of shelf-life at the anticipated storage temperature would be made based on accelerated stability data. It was further noted in this study that the energy of spray-dried amorphous drugs was less than that of the freeze-dried solid by about 2 kcal/mole. It was postulated that the spray-dried drug represents an annealed form of the amorphous drug. In this study, discoloration of the amorphous drug could be detected visually

well before loss of potency could be detected by a chemical assay. The glass transition of the amorphous solid was not measured in this study, but was estimated using the general relationship that $T_g/T_m = 0.6$ (19). The observed nonlinear Arrhenius plot was consistent with decreased activation energy above the T_g of the drug.

Duddu and Weller (16) measured the stability of a freeze-dried aspirin formulation in the region of T_g and pointed out the significant error introduced by measuring the degradation rate above T_g and extrapolating to temperatures below T_g . For a system with a T_g of about 36°C, extrapolation of 40°C, 45°C, and 50°C data yielded a predicted rate constant of 0.009 day⁻¹ at 22°C, whereas the observed value was 0.003 day⁻¹.

Carstensen and Morris (10) studied chemical stability of amorphous indomethacin, largely to determine whether solid-state stability can be determined by measurements of stability in the "molten" state, that is, above the glass transition temperature. Differences in decomposition rate between crystalline (MP 162°C) and amorphous drug at 145°C was approximately an order of magnitude. Arrhenius plots of the decomposition rate of molten solid and the amorphous solid were linear, indicating that chemical stability of the amorphous solid can be predicted by measurement of decomposition rate in the molten state. This finding, of course, is only consistent with "strong glass" behavior of amorphous indomethacin. Decomposition of crystalline indomethacin did not follow first-order kinetics, but rather a Bawn decomposition model (an S-shaped plot of fraction of drug remaining vs. time), which follows the equation $\ln [1-ax] = -[Ak_s]t$, where x is the fraction decomposed and a is an iterant parameter that imposes linearity and zero intercept on the data. This underscores the importance of understanding that the kinetics of decomposition of amorphous and crystalline solids can be quite different, and a detailed study of both mechanism and kinetics of decomposition is needed in order to make meaningful stability predictions from accelerated stability data.

Ball (5) studied the solid-state hydrolysis of single crystals of aspirin, and also observed S-shaped plots of fraction decomposed versus time, but found that the kinetic data fit an Avrami-Erofeyev model involving nucleation at dislocations in the crystal lattice.

ANNEALING TO IMPROVE CHEMICAL STABILITY OF AMORPHOUS FREEZE-DRIED DRUGS

Annealing of amorphous freeze-dried solids below the T_g of the formulation should result in structural relaxation to a lower enthalpy, and more stable, state. The relationship between structural relaxation time and solid-state stability was studied by Abdul-Fattah and coworkers (1) using moxalactam in a 12% mannitol formulation. The main degradation pathway in the solid state is decarboxylation. Structural relaxation time was measured by isothermal calorimetry. The freeze-dried solid was annealed at 60°C, 70°C, and 80°C for varying periods of time. As annealing times and temperatures increased, relaxation times also increased. The rate of decarboxylation for a sample annealed at 70°C for eight hours was roughly 1.7 times lower than the unannealed control. In this case, molecular motion relevant to stability and structural relaxation are coupled.

Wang and Pikal (50) examined the influence of annealing of the freeze-dried solid on stability using sodium ethacrylate as a model drug in both sucrose and trehalose. The main degradation product from the solid state is a dimer. Enthalpy recovery was measured by DSC, and free volume was estimated from density measurements. Global mobility was measured by isothermal calorimetry, and fast local mobility was measured using neutron backscattering. Annealed samples exhibited longer structural relaxation times than the unannealed control, as expected, meaning decreased global mobility. Annealing did not significantly influence local mobility. Stability data at 40°C shows that dimer formation follows square root of time kinetics. Samples annealed at 65°C for 10 hours showed a degradation rate constant of 4.6 %-month^{-0.5}, compared with 7.0 %-month^{-0.5} for the unannealed control. Again, in this case, stability correlates with global mobility, and not with local mobility.

Luthra and coworkers (32) studied the relationship between chemical stability, global molecular motion, and microscopic local mobility in the systems sucrose/aspartame and trehalose/aspartame. The objective was to determine whether annealing of the freeze-dried solid affects its chemical stability as well as to determine whether chemical degradation is coupled with global relaxation times (using calorimetry) and/or with T_1 and $T_1(\rho)$ relaxation times as determined by solid state nmr. These researchers found that a stabilization affect was observed, and that this effect correlated with global mobility.

These studies point to annealing as a potentially useful strategy for improving stability of amorphous freeze-dried solids. However, the development scientist should be alert to the possibility that the annealing process itself may cause a significant increase in degradation product.

THE INFLUENCE OF FORMULATION AND PROCESSING FACTORS ON THE PHYSICAL STATE OF THE DRUG

The physical state of a drug in a freeze-dried solid is influenced not only by the tendency to crystallize from a freezing solution, but also by processing conditions and by other components of the formulation. From a processing standpoint, the most important parameter may be the thermal history of freezing. From a formulation perspective, the relative amount of drug in the formulation influences the ability of the drug to crystallize during the time frame of freezing and freeze drying, as well as the nature of other formulation components.

Yarwood and coworkers (51) investigated the influence of cooling rate, initial concentration of solute, and fill volume on physical form and chemical stability of sodium ethacrylate. The freezing protocol consisted of ramping from room temperature to -25°C over four hours versus placing vials on shelves precooled to about -50°C . Drug concentration varied from 0.5% to 4% (w/v), and fill volume varied from 0.5 to 3 mL. Accelerated stability testing of freeze-dried solids was carried out at 60°C . Rapidly cooled samples were amorphous to x-rays, whereas slowly cooled samples were crystalline. Striking differences were observed in stability of the drug, depending on physical state. For crystalline sodium ethacrylate, 95% of the drug remained after 90 days. Amorphous drug prepared from a 1% solution at a fill volume of 0.5 mL degraded by more than half after 60 days. While no mention was made in this study of either T_g' or T_g (the glass transition temperature of the freeze-dried solid), it was noted that some samples "liquefied to produce a clear oil" at the stress testing condition, which indicates that the test was carried out above T_g of the amorphous solid. This is consistent with the DSC thermogram of amorphous sodium ethacrylate in Figure 8, showing the glass transition at about 60°C . Accelerated stability data do show that higher fill volumes result in a slower effective rate of freezing as reflected by the higher relative amount of amorphous material. For a starting solution concentration of 1% and a fill volume of 0.5 mL, only 42% of drug remained after 60 days at 60°C . For a fill volume of 3 mL and the same starting drug concentration, 79% of intact drug remained after the same time interval. This is consistent with slower freezing of higher fill volumes resulting in a higher percentage of crystalline material. Higher concentrations of drug, as expected, favor crystallization. Increasing from 1% drug to 4% favors crystallization, as reflected by 79% of drug remaining after 60 days at 60°C versus 42% remaining for freeze-dried solid resulting from a 1% solution.

Oguchi and coworkers (35) examined the decarboxylation behavior of *p*-aminosalicylic acid (PAS) as a freeze-dried solid under accelerated conditions (80°C) in the presence of the excipients pullulan (a linear polysaccharide, which cannot form inclusion complexes) and α -cyclodextrin. The solid-state stability was shown to correlate with the fraction of amorphous PAS. Increasing relative amounts of pullulan resulted in higher fractions of amorphous PAS. Rapid freezing (liquid nitrogen) was shown to result in a greater relative amount of amorphous drug, as expected. Solid-state stability of PAS as a function of molar ratio of α -cyclodextrin (CD) to PAS was complex. At low ratios of CD to PAS, stability decreased because of inhibition of crystallization of PAS during freezing. Higher molar ratios stabilized the PAS because of formation of a crystalline inclusion complex. Again, rapid freezing resulted in higher rates of decarboxylation in the solid state.

Residual Moisture Effects

In studying the relationship between T_g and stability, it is important to recognize that water can serve not only as a plasticizer of the amorphous phase, but also as a reactant. Bell and Hageman (7) used a freeze-dried preparation of aspartame in different molecular weights of poly(vinylpyrrolidone) in order to address the question of which is more important—the glass transition temperature or residual water activity. The solid-state degradation of aspartame via rearrangement to a diketopiperazine was measured at 25°C after equilibrating the model formulations containing different molecular weights of PVP at different relative humidities. It was found that reaction rates at constant water activity, but different T_g values, were not significantly different. However, rates at similar $(T - T_g)$ values, but different water activities, were significantly different. Therefore, in this system, water activity is more important than $(T - T_g)$.

It is important to establish the degree to which residual moisture affects stability of a freeze-dried solid and to have data to support a residual moisture specification. One way to do this is to equilibrate the freeze-dried product at a range of relative humidities using saturated salt solutions. Of course, the relationship between relative humidity and residual moisture must be established, but a relative humidity range from about 6% to perhaps 20% often works well. Once an appropriate range is established, these subbatches are placed on stability, often under stressed conditions.

EXCIPIENT EFFECTS ON DRUG STABILITY IN FREEZE-DRIED DOSAGE FORMS

Crystallization of excipients from freeze-dried solids can affect stability of drugs as freeze-dried solids. Herman and coworkers (26) studied the solid-state stability of methylprednisolone

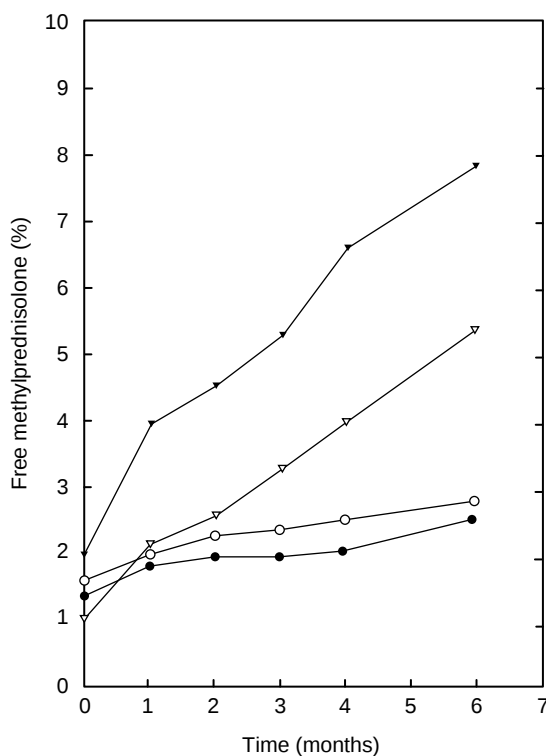


Figure 11 Rate of appearance of methylprednisolone, the hydrolysis product of methylprednisolone sodium succinate during stress testing at 40°C: control using 250mg drug per vial with no excipient (closed circles), 40mg of drug in 210mg lactose (open circles), 125mg drug in 125mg mannitol (open triangles), and 40mg drug in 210mg mannitol (closed triangles).

sodium succinate in the presence of the bulking agents mannitol and lactose. Comparative stability data at 40°C are shown in Figure 11, where the rate of appearance of the hydrolysis product, methylprednisolone, is considerably faster for mannitol than for lactose, despite similar residual moisture levels. X-ray diffraction analysis at intervals during the stability study shows that the freeze-dried solid containing mannitol is initially amorphous, but that mannitol crystallizes during storage in the solid state (Fig. 12). The formulation containing lactose as a bulking agent remains amorphous throughout the stability study. The most likely explanation for this observation is that mannitol crystallization affects the distribution of residual moisture in the solid; that is, as mannitol crystallizes, it excludes water from the crystal lattice, thereby increasing the level of residual moisture in the remaining amorphous phase and increasing the rate of hydrolysis. This effect is enhanced by a small, but significant, increase in residual moisture during storage due to the transfer of water vapor from the rubber stopper, where this additional water is localized in the amorphous phase, thereby further increasing the water activity in the microenvironment of the drug. Representative data for residual moisture as a

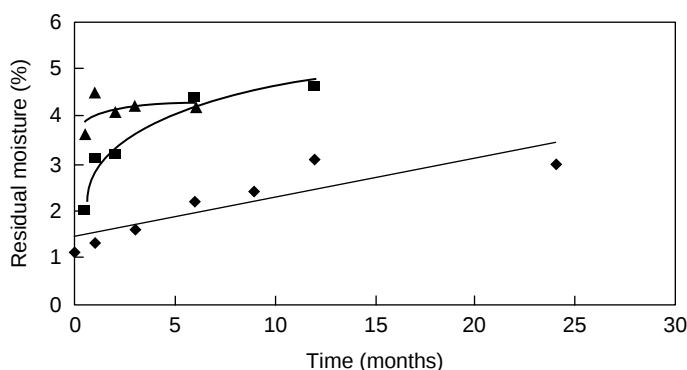


Figure 12 Increase in residual moisture of a freeze-dried solid resulting from water vapor transfer from the elastomeric closure as a function of storage temperature: 40°C (triangles), 25°C (squares), and 5°C (diamonds).

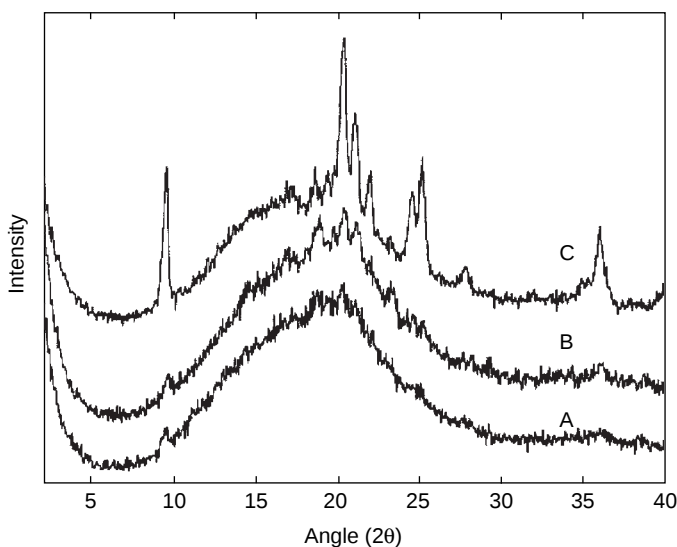


Figure 13 X-ray powder diffractograms of methylprednisolone sodium succinate in mannitol during stress testing at 40°C, showing crystallization of mannitol during storage: (A) initial freeze-dried powder, (B) two months, and (C) six months.

function of time at different temperatures are shown in Figure 13. As expected, the residual moisture level increases more rapidly at higher temperature, but the plateau level is independent of temperature as equilibrium is established between the freeze-dried solid and the stopper. The extent to which this is observed depends on several factors. First, the nature of the rubber stopper formulation affects the diffusivity of water in the rubber. Second, the processing of the stopper can affect the level of residual moisture present. It is not uncommon for extended drying of the stopper to be necessary to minimize residual moisture (15). Finally, the mass of the freeze-dried solid determines the extent to which the percent residual moisture is affected by water vapor transfer from the stopper, where large cakes may be relatively unaffected by the small amount of water vapor that is transferred from the stopper. Two lessons can be learned from this information from the standpoint of design of stability studies. First, it is important to be aware of potential changes in physical state during storage for both drug and excipients, and to monitor the physical state of freeze-dried solids periodically during stability studies. Second, residual moisture should be monitored periodically, particularly for relatively low-mass solids, because of the potential for water vapor transfer from stopper to product.

A sometimes important difference between the freeze drying of proteins and the freeze drying of small molecules is that quality attributes of freeze dried protein formulations are more often affected in measurable ways by differences in thermal history of freezing relative to small-molecule formulations. However, differences in quality attributes of small molecule products caused by differences in thermal history of freezing have been shown to occur. Chongprasert and coworkers (1998) investigated the photostability of freeze-dried pentamidine isethionate produced at different concentrations of drug in the initial solution as well as different thermal histories of freezing. Polymorph screening revealed three anhydrites, designated A, B, and C, as well as a trihydrate (Fig. 14). Form C is a high-temperature form, and cannot be

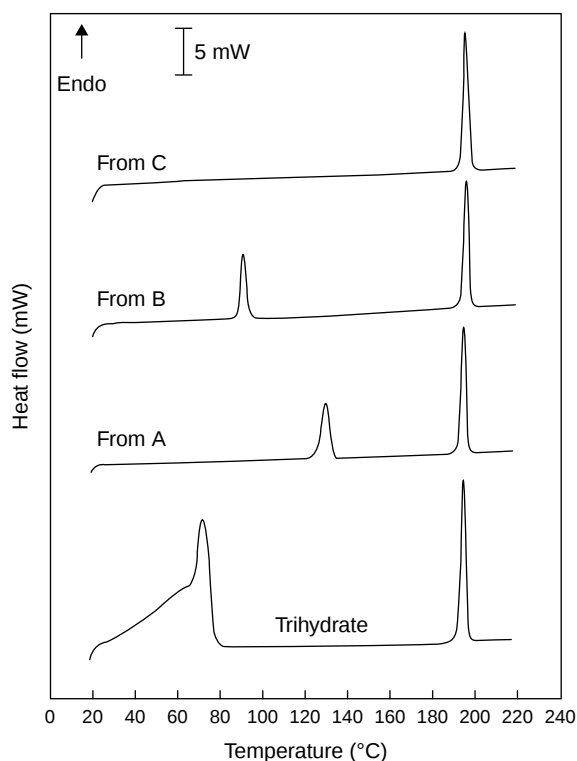


Figure 14 DSC thermograms of different crystal forms of pentamidine isethionate.

produced under lyophilization conditions. At concentrations of drug of 4% (w/v) or less, Form A is produced regardless of the freezing method used. At concentrations of 10%, the crystal forms observed are a function of the freezing method. The freezing methods examined were 1) cooling on the shelf at 2°C and holding for three hours prior to decreasing the shelf temperature to -45°C, 2) directly cooling on the shelf from room temperature to -45°C, 3) quench-cooling in liquid nitrogen. Results showed that form A, form B, or a mixture of both forms, are present in the freeze-dried solid depending on whether the trihydrate crystallizes during freezing or not. Form B can only be produced by dehydration of the trihydrate at low temperature. Photostability studies over a period of 2 weeks at 1000 ft-candles demonstrated that form B remained a white powder whereas form A turned distinctly pink after about 1 week. The results of the study underscore the point that validation studies to identify critical process variables should include thermal history of freezing, even for small molecule formulations.

STABILITY OF FREEZE-DRIED PROTEIN FORMULATIONS

Loss of integrity of proteins as freeze-dried solids falls into two broad categories—physical instability and chemical instability. Physical instability does not involve making or breaking of covalent bonds, rather it refers to changes in higher order structure (secondary, tertiary, or quaternary) of proteins, where hydrophobic regions, normally “buried” inside the folded conformation, are exposed. Resulting hydrophobic interactions between proteins can cause formation of noncovalent aggregates (either soluble or insoluble). A common problem in freeze drying of proteins is turbidity, or haze, in reconstituted solutions, arising from insoluble aggregates. Chemical instability involves covalent modification of the primary structure via bond cleavage or bond formation. Examples of chemical instability include covalent aggregation (usually through disulfide bond formation), deamidation, oxidation, hydrolysis, or nonenzymic browning (Maillard reaction) (see also chap. 14). These two types of instability are not entirely independent, since protein unfolding (physical instability) can result in increased rates of chemical instability.

It is important, of course, to distinguish between short-term stability and long-term stability in the context of freeze drying of protein pharmaceuticals, where short-term stability refers to maintenance of protein integrity throughout the freezing and freeze-drying stages of the process. Stability against damage caused by the freeze-dry process itself usually involves physical stability, where the protein partially unfolds during the process, exposing hydrophobic residues such that, upon reconstitution, aggregates are formed. It is important to “break down” the process, and determine where the damage is taking place. Unlike small molecules, proteins can be irreversibly damaged by the freezing process, so examining stability against freezing damage is a logical first step in developing freeze-dried protein pharmaceuticals, keeping in mind that appropriate protectants against freezing damage may be different than protects against damage caused by drying.

THE POTENTIAL ROLE OF ICE-PROTEIN INTERACTIONS IN DAMAGE INDUCED BY FREEZE DRYING

There are several mechanisms by which proteins can be damaged by the effects of freezing. These include the effect of freeze concentration, effects of ionic strength, and potential pH shifts in partially frozen systems as water is removed by ice crystallization. However, there is a small but growing body of evidence that an important mechanism underlying such short-term instability is the accumulation of protein at the ice/freeze-concentrate interface during freezing. This is probably not due to specific ice/protein interactions, although such interactions certainly exist, but rather an entropically driven process where protein partly unfolds at the ice/freeze concentrate interface during freezing. There is a boundary layer of water a few molecules thick at the ice surface; in other words, water does not proceed from being completely rigid in the ice to completely mobile at the ice surface. When a protein unfolds at the surface, the entropy of the system increases because of the water that is “freed-up” as a result of the

adsorption process. While some configurational entropy of the protein is lost, the fate of the water is much more important on a molar basis. The unfolding of the protein may be at least partly reversible upon freeze thawing but, if the system is freeze dried, proteins are more likely to get “stuck” in the partly unfolded conformation, leading to aggregation upon reconstitution.

Supporting evidence for this mechanism includes fluorescence lifetime measurements by Strambini and Gabellieri (44) consistent with ice-induced partial unfolding. Bhatnagar and co-workers (6) compared the effects of freeze concentration with and without ice present using lactate dehydrogenase under isothermal conditions. While negligible degradation occurred in the concentrated solutions, massive degradation was observed with ice present. Recently, Schwegman and coworkers (43) used FTIR microscopy to demonstrate partial unfolding of model proteins in the microenvironment of ice crystals in partially frozen systems. No evidence of partial unfolding was observed in the interstitial space between ice crystals.

This mechanism also explains much of the phenomenology associated with freeze drying of proteins. For example, it is generally recognized that stability against damage associated with freeze drying of proteins increases as protein concentration increases (40). As protein concentration increases, a point is reached where the ice/freeze concentrate interface is saturated with protein, and additional protein is protected from the interface. Rapid freezing is generally bad for acute stability of proteins (17,27), because rapid freezing promotes increased ice/freeze concentrate interfacial area. In addition, surfactants commonly have a protective effect, where this could arise from competitive inhibition of protein adsorption at the interface by the surfactant.

LONG-TERM STABILITY OF PROTEINS AS FREEZE-DRIED SOLIDS

Once challenges in maintaining stability against acute damage caused by freeze-drying have been overcome, there remains a challenge in maintaining integrity during storage of the freeze-dried solid. Of course, ice/protein interactions have no direct role in this type of stability other than to affect initial recovery of activity. As discussed above, it is common to use stabilizers—typically sugars and polyols—to stabilize proteins against the stresses involved in freezing and dehydration. Generally speaking, disaccharides are the most effective, and most widely used, stabilizers. There are two hypotheses as to how these stabilizers work—one involving thermodynamic stabilization, and the other involving kinetic stabilization.

The “water substitute hypothesis” refers to thermodynamic stabilization by hydrogen bonding between the sugar or polyol and the protein. Water hydrogen bonds to a protein in its native environment, and this hydrogen bonding is critical in maintaining the thermodynamic stability of the protein. As water is removed by drying, the equilibrium shifts toward the unfolded conformation. Sugars and polyols are believed to stabilize by acting as a surrogate for the water, thus shifting equilibrium back toward the native conformation. Thus, the native conformation is maintained by thermodynamic stabilization of the native conformation. Practically speaking, infrared spectroscopy is probably the most useful method for measuring the extent to which the native conformation is maintained in the freeze-dried solid. The details are beyond the scope of this chapter, and the reader is referred to reviews by Carpenter and co-workers (9). A more recent work points to the potential utility of near-infrared spectroscopy as a noninvasive tool for measuring the degree of native conformation in proteins (4).

The alternative hypothesis of how stabilizers work is kinetic stabilization of the protein in a glassy matrix. The rigid matrix in which the protein is dispersed thus prevents significant motion, and the lack of motion confers stability to the protein. This is referred to in older literature as the “vitrification hypothesis,” where the thermodynamically preferred state is irrelevant, because the protein is trapped in the glassy matrix. Stabilizers with high T_g relative to the storage temperature are said to be good glass formers. As discussed above, though, the glass transition temperature is only part of the story. The concept of “strong” and “fragile” glasses comes into play, where this refers to the temperature dependence of molecular mobility in the

region of the glass transition. A “strong” glass resists structural change and exhibits near Arrhenius-like behavior in its relationship between molecular mobility and temperature. Strong glasses also exhibit small changes in heat capacity at T_g . In fragile glasses, on the other hand, molecular mobility changes much more dramatically with temperature in the region of T_g , and the change in heat capacity at T_g is relatively large. Thus, when we consider two different formulations with different T_g s, it is possible for the formulation with the higher T_g to have higher molecular mobility at the storage temperature.

Methods for studying glass dynamics include scanning calorimetry, isothermal calorimetry, NMR, dielectric spectroscopy, neutron scattering, and a newer calorimetric technique known as thermally stimulated current (TSC). The types of motion measured by these methods are different. Enthalpy relaxation as measured by DSC refers primarily to translational motion, and is generally assumed to be equivalent to the motion associated with viscous flow. This type of mobility has relaxation times on the order of tens to hundreds of seconds. NMR and dielectric spectroscopy measure faster, mostly rotational motion. Calorimetric methods for measuring structural relaxation in amorphous materials have been reviewed by Kawakami and co-workers (29).

Of course, a key question here is “what type of motion is relevant to the long-term stability of proteins?” This has been the subject of some important investigations in recent years. Chang and co-workers (11,12) examined the stability of an IgG1 antibody in either sucrose or trehalose alone or in combination with sorbitol. Aggregation and chemical stability were monitored by size exclusion chromatography and by ion exchange chromatography, respectively; and structural relaxation time (τ) was measured by isothermal calorimetry. Addition of a small amount of sorbitol to a sucrose-based formulation resulted in greater retention of native structure, faster structural relaxation, but improved stability. Addition of sorbitol to the trehalose-based formulation resulted in no change in native structure, but the decrease in relaxation time and the improvement in stability were similar to that of the sucrose-based formulation. Thus, glass dynamics (as measured by τ) did not explain the stability results for either formulation. Stability correlated best with native structure for the sucrose formulation but, for the trehalose-based formulation, neither native structure nor glass dynamics explained the stability results. It was speculated that, in this case, alpha motion does not correlate with stability, but beta motion would, and the influence of sorbitol is on beta-type motion. These investigators also observed that the best stability was observed when residual moisture was in the range of 2–3%, which is consistent with the idea that, concerning the long-term stability of proteins, dryer is not always better.

Katayama and coworkers (28) carried out a retrospective analysis of 18 lyophilized progenipointin formulations using multivariate statistical analysis in order to determine the parameters critical to stability. Stability correlated best with retention of native structure and with pH of the pre-freeze-dried formulation. T_g was not a strong predictor of stability, where all of the formulations had T_g values above the storage temperature of 40°C.

Yoshioka and colleagues (52,54) studied the temperature dependence of the stability of insulin freeze dried with poly(vinylpyrrolidone) at temperatures both above and below T_g of the formulation. Solid-state degradation was via 21-desamido formation and dimerization. Degradation rate followed first order kinetics, and plots of t_{90} versus T/T_g were linear, with no change in slope around T_g . This is consistent with the conclusion that molecular mobility as reflected by T_g is not an important determinant of stability. Subsequent work by these same investigators (55) on stability of insulin lyophilized with either trehalose or dextran explored beta relaxation, as measured by the spin-lattice relaxation time (T_1) of the insulin carbonyl carbon as an indicator of stability. The degradation rate of insulin was not significantly affected by dextran, and dextran had no influence on T_1 . Trehalose, on the other hand, both stabilized insulin and prolonged T_1 at low humidity. The authors conclude that beta relaxation is more important than molecular mobility of the matrix in determining stability in this system.

Yoshioka et al. (53) examined aggregation of β -galactosidase when freeze dried with either sucrose, trehalose, or stachyose. Again, molecular mobility as measured by T_g was compared with beta relaxation as measured by the spin-lattice relaxation time of the carbonyl carbon of the protein. The glass transition temperatures of the matrices followed the order sucrose<trehalose<stachyose. In this case, the aggregation rate of the formulations changed slope in the region of T_g , but T_g was not a good predictor of stability. In fact, the rates of aggregation at temperatures both above and below T_g followed the order sucrose<trehalose<stachyose. The rank order of these sugars on slowing the spin-lattice relaxation time was sucrose>trehalose>stachyose, thus supporting the idea that aggregation of β -galactosidase is determined more by local mobility than by global mobility of the matrix. Significant differences in native structure between stabilizers, as measured by FTIR, were not observed in this study.

Pikal and coworkers (38) studied storage stability of human growth hormone (hGH, both chemical and physical, as measured by aggregation) in lyophilized disaccharide formulations. Molecular mobility of the matrix was examined by structural relaxation times as measured by DSC, and atomic motion on a nanosecond time scale was measured by neutron scattering. Structure of protein in the solid state was studied by FTIR. For storage well below T_g , stability appeared unrelated to T_g . Stability was only weakly correlated with secondary structure of the protein. At equivalent levels of disaccharide relative to protein, sucrose formulations were about a factor of 2 more stable than trehalose formulations, yet had greater mobility as measured by structural relaxation time. Neutron scattering results showed greater suppression of fast motion by sucrose than by trehalose, suggesting that, at temperatures well below T_g , fast dynamics are important to stability.

Wang and co-workers (49) investigated the impact of sucrose level on storage stability of five different proteins. Each model protein was freeze dried with different amounts of sucrose, and protein aggregation was monitored by size exclusion chromatography. Protein secondary structure was monitored by FTIR, and global mobility was measured by isothermal calorimetry. Fast dynamics was monitored by neutron backscattering. The density of the protein formulations was measured by gas pycnometer. Physical stability of the proteins increased continuously with increasing sucrose level over the range of compositions studied. Both the degree of retention of native structure and structural relaxation measured by calorimetry reached maxima at about 1:1 mass ratios of sucrose to protein for most of the proteins studied, so stabilization cannot be explained by either global dynamics or retention of native structure. However, fast local mobility and free volume measured from density decreased monotonically with increased sucrose level, thus supporting the idea that local dynamics and free volume correlate well with storage stability of the proteins studied.

Meyer and coworkers (33) examined the impact of combinations of sucrose with mannitol or glycine on the stability of a lyophilized monoclonal antibody, focusing on deamidation and aggregation. While sucrose was the main stabilizing agent, the inclusion of some amorphous glycine resulted in a significant increase in stability. Mannitol displayed little, if any, additional stabilizing effect. This effect was observed despite the fact that the addition of glycine causes a decrease in the glass transition of the freeze-dried solid.

These studies point to some general conclusions about stabilizers and long-term stability of proteins. First, sucrose seems to consistently emerge as a stabilizer with a high probability of success in stabilizing proteins as freeze-dried solids. Second, the glass transition temperature of a formulation does not appear to be an effective predictor of protein stability, even on a rank-order basis. Finally, while there are still not enough published data to allow definitive conclusions, it appears that methods that measure fast, local mobility such as solid-state C-13 NMR and neutron backscattering may ultimately emerge as useful predictors of stability.

THE EFFECT OF COLLAPSE ON LONG-TERM STABILITY OF PROTEINS

It is important to distinguish between structural collapse as a cosmetic defect and a defect that could result in subtherapeutic dosing. There is a widely held—but not documented—point of view that structural collapse during freeze drying adversely affects the short-term stability of proteins, the long-term stability, or both. The subject has not been examined extensively, but an

investigation by Wang and co-workers (48) on freeze-dried recombinant factor VIII and α -amylase, where matrices were deliberately collapsed during freeze drying and placed on stability, showed no adverse effect of collapse.

Schersch and coworkers (41) investigated the influence of structural collapse on the short-term stability of a monoclonal IgG, antibody as well as lactate dehydrogenase. They found that collapsed cakes had comparable residual moisture levels to noncollapsed cakes, reconstitution times were not increased for collapsed cakes, and protein integrity after freeze drying was not adversely affected by structural collapse.

The above studies should be regarded with some caution, however, because structural collapse does decrease the specific surface area of the freeze-dried solids, and this commonly results in elevated levels of residual moisture. Elevated residual moisture levels are commonly a “warning flag” for potential stability problems.

CONCLUSION

Freeze-dried parenteral dosage forms generally offer a pharmaceutically elegant alternative to solution formulations when the drug is unstable in solution. However, it is important to understand that freeze-dried solids are not always physically and chemically stable enough to support a practical shelf-life, and that the physical state of a freeze-dried solid is affected by the nature of the solute, by the thermal history of freezing, and by interaction with other components of the formulation. Characterization of the freeze-dried solids in order to determine the physical state, that is, crystalline or amorphous, is an essential element of both formulation and process development. Knowledge of the glass transition temperature of an amorphous solid, as well as how the glass transition is affected by the level of residual moisture, is essential in order to define meaningful stress testing conditions. For crystalline solids, it is important to know whether different polymorphs exist and, if so, the relative stability of these polymorphs. Monitoring for changes in physical state of the solids should be included in stress testing.

For freeze-dried proteins, it is important to be aware of fundamental differences between small molecules and proteins in the context of freeze drying, including potential susceptibility to freezing-induced damage as well as the possibility that overdrying could be damaging to protein integrity. For both small molecules and proteins, it may be useful to stay informed about research relating different kinds of molecular mobility to solid-state stability with an eye toward development of screening tools to make the pharmaceutical scientist less dependent on real-time stability measurement as a rate-limiting step in product development.

The following table summarizes some key features of the product and the process with respect to potential product quality concerns, including stability.

	Attribute	Methodology	Comments
Formulation	Maximum allowable product temperature during primary drying	Low temperature thermal analysis and freeze dry microscopy	Collapse temperatures lower than about -35°C often cause problems in scale-up. Discussed in Materials Characterization section.
Freeze-drying	Freezing process	Monitor temperature in vials of product	Aspects of freezing to be considered include the temperature ramp rate of shelves as well as the shelf temperature at which product is loaded. Discussed in The Influence of Formulation and Processing Factors on the Physical State of the Drug.

(Continued)

(Continued)

	Attribute	Methodology	Comments
Primary drying	Monitor temperature in vials of product	Product collapse (amorphous solute) and "melt-back" (eutectic melting) are of particular concern, as well as shrinkage of the cake. Discussed in the Process Overview section as well as in Materials Characterization.	
Secondary drying	Monitor temperature in vials of product	Collapse can take place during secondary drying if shelf temperature is increased too rapidly. Discussed in Materials Characterization section.	
Final product	Container/closure integrity	Measurement of headspace pressure; FM spectroscopy	This applies to lyophilized solids subject to oxidative degradation in the solid state. Discussed in the Process Overview section.
	Physical state of freeze-dried solid	X-ray diffraction; thermal analysis	For amorphous solids, measure T_g as a function of residual moisture content. For crystalline solids, identify the crystal form present. Discussed in Materials Characterization section.
	Physical stability	X-ray diffraction; thermal analysis	Monitor for changes in physical state, particularly amorphous to crystalline transitions.
	Appearance of freeze-dried solid	Visual inspection	The solid should have the same size and shape as the liquid originally filled into the vial. Should have uniform color and texture.
	Chemical stability	A suitable stability-indicating assay	For amorphous or partially amorphous systems, the storage temperature should be well below the T_g of the freeze-dried solid.
Reconstituted solution	Reconstitution time	Visual inspection	Should be no more than about 3 min
	Appearance of reconstituted solution	Visual inspection	Should be clear, with no haze or visible particulate matter
	Reconstituted stability	Suitable potency-indicating assay and visual inspection	Reconstituted stability seldom needs to be longer than 48 hr.

REFERENCES

1. Abdul-Fattah AM, Dellerman KM, Bogner RH, Pikal MJ. The effect of annealing on the stability of amorphous solids: chemical stability of freeze-dried moxalactam. *J Pharm Sci* 2007; 96:1237–50.
2. Akers MJ, Milton N, Byrn SR, Nail SL. Glycine crystallization during freezing: Effects of salt form, pH, and ionic strength. *Pharm Res* 1995; 12:1457–61.
3. Arakawa T, Prestrelski SJ, Kenney WC, Carpenter JF. Factors affecting short-term and long-term stability of proteins. *Adv Drug Delivery Reviews* 2001; 46: 307–26.

4. Bai S, Nayar R, Carpenter JF, Manning MC. Noninvasive determination of protein conformation in the solid state using near infrared (NIR) spectroscopy. *J Pharm Sci* 2005; 94: 2030–8.
5. Ball MC. Solid-state hydrolysis of aspirin. *J Chem Soc Faraday Trans* 1994; 90:997–1001.
6. Bhatnagar BS, Pikal MJ, Bogner RH. Study of the individual contributions of ice formation and freeze-concentration on isothermal stability of lactate dehydrogenase during freezing. *J Pharm Sci* 2008; 97: 798–814.
7. Bell LN, Hageman MJ. Differentiating between the effects of water activity and glass transition dependent mobility on a solid-state chemical reaction: aspartame degradation. *J Agric Food Chem* 1994; 42: 2398–401.
8. Cannon AJ, Trappler EH. The influence of lyophilization on the polymorphic behavior of mannitol. *PDA J Pharm Sci Tech* 2000; 54: 13–22.
9. Carpenter JF, Prestrelski SL, A Dong. Application of infrared spectroscopy to development of stable lyophilized protein formulation. *Eur J Pharm Biopharm* 1998; 45: 231–8..
10. Carstensen JT, Morris T. Chemical stability of indomethacin in the solid amorphous and molten states. *J Pharm Sci* 1993; 82: 657–9.
11. Chang L, Shepherd D, Sun J et al. Mechanism of protein stabilization by sugars during freeze-drying and storage: native structure preservation, specific interaction, and/or immobilization in a glassy matrix? *J Pharm Sci* 2005; 94: 1427–44.
12. Chang L, Shepherd D, Sun J, Tang XC, Pikal MJ. Effect of sorbitol and residual moisture on the stability of lyophilized antibodies: Implications for the mechanism of protein stabilization in the solid state. *J Pharm Sci* 2005; 94: 1445–55.
13. Chongprasert S, Knopp SA, Nail SL. Characterization of frozen solutions of glycine. *J Pharm Sci* 2001; 90: 1720–8.
14. Chongprasert S, Griesser UJ, Böttorff AT, Byrn SR, Nail SL. Effects of process conditions on crystallization of pentamidine isethionate during freeze drying. *J Pharm Sci* 1998; 87: 1155–60.
15. Donovan PD, Corvari V, Burton MD, Rajagopalan N. Effect of stopper processing conditions on moisture content and ramifications for lyophilized products: comparison of “low” and “high” moisture uptake stoppers. *PDA J Pharm Sci Tech* 2007; 61: 51–8.
16. Duddu SP, Weller K. Importance of glass transition temperature in accelerated stability testing of amorphous solids: case study using a lyophilized aspirin formulation. *J Pharm Sci* 1966; 85: 345–7.
17. Eckhardt BM, Oeswein JQ, Tewley TA. Effect of freezing on aggregation of human growth hormone. *Pharm Res* 1991; 8: 1360–4.
18. Gomez G, Pikal MJ, Rodriguez-Hornedo N. Effect of initial buffer composition on pH changes during far-from-equilibrium freezing of sodium phosphate buffer solutions. *Pharm Res* 2001; 18: 90–7.
19. Hancock BC, Zografi G. Characteristics and significance of the amorphous state in pharmaceutical systems. *J Pharm Sci* 1997; 86: 1–11.
20. Hancock BC, Dalton CR, Pikal MJ, Shamblin SL. A pragmatic test of a simple calorimetric method for determining the fragility of some amorphous pharmaceutical materials. *Pharm Res* 1998; 15: 762–7.
21. Hatley RHM, Franks F, Day H, Byth B. Subzero temperature preservation of reactive fluids in the undercooled state: I. The reduction of potassium ferricyanide by potassium cyanide. *Biophys Chem* 1986; 24: 41–6.
22. Hatley RHM, Franks F, Day H. Subzero temperature preservation of reactive fluids in the undercooled state II. The effect on the oxidation of ascorbic acid of freeze concentration and undercooling. *Biophys Chem* 1986; 24: 187–92.
23. Hawe A, Friess W. Impact of freezing procedure and annealing on the physico-chemical properties and the formation of mannitol hydrate in mannitol-sucrose-NaCl formulations. *Eur J Pharm Biopharm* 2006; 64: 316–25.
24. Hemminga MA, Roozen M, Walstra P. Molecular motions and the glassy state. In: Blanshard JMV, Lillford PJ, eds. *The Glassy State in Foods*. , Loughborough, Leicestershire: Nottingham University Press, 1993: 157–71.
25. Her LM, Nail SL. Measurement of glass transition temperatures in freeze concentrated solutions by differential scanning calorimetry. *Pharm Res* 1994; 11: 54–9.
26. Herman BD, Sinclair BD, Milton N, Nail SL. The effect of bulking agent on the solid-state stability of freeze-dried methylprednisolone sodium succinate. *Pharm Res* 1994; 11: 1467–73.
27. Jiang S, Nail SL. Effect of process conditions on recovery of protein activity after freezing and freeze-drying. *Eur J Pharm Biopharm* 1998; 45: 249–57.

28. Katayama DS, Kirchhoff CF, Elliott CM et al. Retrospective statistical analysis of lyophilized protein formulations of progenipoietin using PLS: determination of the critical parameters for long-term storage stability. *J Pharm Sci* 2004; 93: 2609–23.
29. Kawakami K, Pikal MJ. Calorimetric investigation of the structural relaxation of amorphous materials: evaluating validity of the methodologies. *J Pharm Sci* 2005; 94: 948–65.
30. Kim AI, Knopp S, Akers MJ, Nail, SL. The physical state of mannitol after freeze-drying: effects of mannitol concentration, freezing rate, and a non-crystallizing cosolute. *J Pharm Sci* 1998; 87: 931–5.
31. Larsen SS. Studies on stability of drugs in frozen systems: VI. The effect of freezing upon pH for buffered aqueous solutions. *Arch Pharm Chem* 1973; 1: 433–5.
32. Luthra SA, Hodge IM, Utz M, Pikal MJ. Correlation of annealing with chemical stability in lyophilized pharmaceutical glasses. *J Pharm Sci* 2008; 97: 5240–51.
33. Meyer JD, Nayar R, Manning MC. Impact of bulking agents on the stability of a lyophilized monoclonal antibody. *Eur J Pharm Sci* 2009; 38: 29–38.
34. Nunes C, Suryanarayanan R, Botez CE, Stephens PW. Characterization and crystal structure of D-mannitol hemihydrate. *J Pharm Sci* 2004; 93: 2800–9.
35. Oguchi T, Yonemochi E, Yamamoto K, Nakai Y. Freeze-drying of drug-additive binary systems: II. Relationship between decarboxylation behavior and molecular states of p-aminosalicylic acid. *Int J Pharm* 1990; 61: 27–34.
36. Pikal MJ, Lukes AL, Lang JE. Thermal decomposition of amorphous β -lactam antibacterials. *J Pharm Sci* 1977; 66: 1312–16.
37. Pikal-Cleland KA, Rodriguez-Hornedo N, Amidon GL, Carpenter J F. Protein denaturation during freezing and thawing in phosphate buffer systems: monomeric and tetrameric beta-galactosidase. *Arch Biochem Biophys* 2000; 384: 398–406.
38. Pikal MJ, Rigsbee D, Roy ML, Galbreath D et al. Solid state chemistry of proteins: II. The correlation of storage stability of freeze-dried human growth hormone (hGH) with structure and dynamics in the glassy solid. *J Pharm Sci* 2008; 97: 5106–21.
39. Pincock RE, Kiovsky TE. Kinetics of reactions in frozen solutions. *J Chem Ed* 1969; 43: 358–60.
40. Sarciaux JM, Mansour S, Hageman MJ, Nail SL. Effects of buffer composition and processing conditions on aggregation of bovine IgG during freeze-drying. *J Pharm Sci* 1999; 88: 1354–61.
41. Schersch K, Betz O, Muelau S, Bassarab S, Winter G. Systematic investigations of the effect of lyophilization collapse on pharmaceutically relevant proteins: I. Stability after freeze drying. *J Pharm Sci* 2010; 99: 2256–78.
42. Schuster M, Aaviksaar A, Haga M, et al. Protease-catalyzed peptide synthesis in frozen aqueous systems: the “freeze concentration” model. *Biomed Biochim Acta* 1991; 50: S84–9.
43. Schwegman JJ, Carpenter JF, Nail SL. Evidence of partial unfolding of proteins at the ice/freeze concentrate interface by infrared microscopy. *J Pharm Sci* 2009; 98: 3239–48.
44. Strambini GB, Gabellieri E. Proteins in frozen solutions: evidence of ice-induced partial unfolding. *Biophys J* 1996; 70: 971–6.
45. Sundarmurthy P, Shalaev E, Suryanarayanan R. Calorimetric and diffractometric evidence for the sequential crystallization of buffer components and the consequential pH swing in frozen solutions. *J Phys Chem* 2010; 114: 4915–23.
46. van den Berg L. The effect of addition of sodium and potassium chloride to the reciprocal system: KH_2PO_4 – Na_2HPO_4 – H_2O on pH and composition during freezing. *Arch Biochem Biophys* 1959; 84: 305–15.
47. van den Berg L, Rose D. Effect of freezing on the pH and composition of sodium and potassium phosphate solutions: The reciprocal system KH_2PO_4 – Na_2HPO_4 – H_2O . *Arch Biochem Biophys* 1959; 81: 319–29.
48. Wang DQ, Hey JM, Nail SL. Effect of collapse on the stability of freeze-dried recombinant factor VIII and alpha-amylase. *J Pharm Sci* 2004; 93: 1253–63.
49. Wang B, Tchessalov S, Cicerone MT, Warne MW, Pikal MJ. Impact of sucrose level on storage stability of proteins in freeze-dried solids: II. Correlation of aggregation rate with protein structure and molecular mobility. *J Pharm Sci* 2009; 98: 3145–66.
50. Wang B, Pikal MJ. The impact of thermal treatment on the stability of freeze dried amorphous pharmaceuticals: I. Dimer formation in sodium ethacrylate. *J Pharm Sci* 2010; 99: 663–82.
51. Yarwood RJ, Phillips AJ, Collett JH. Processing factors influencing the stability of freeze-dried sodium ethacrylate. *Drug Dev Indust Pharm* 1986; 12: 2157–70.
52. Yoshioka S, Aso Y. A quantitative assessment of the significance of molecular mobility as a determinant for the stability of lyophilized insulin formulations. *Pharm Res* 2005; 22: 1358–64.

53. Yoshioka S, Aso Y, Kojima S. Softening temperature of lyophilized bovine serum albumin and γ -globulin as measured by spin–spin relaxation time of protein protons. *J Pharm Sci* 2007; 86: 470–4.
54. Yoshioka S, Aso Y, Miyazaki T. Negligible contribution of molecular mobility to the degradation rate of insulin lyophilized with poly(vinylpyrrolidone). *J Pharm Sci* 2006; 95: 939–43.
55. Yoshioka S, Miyazaki T, Aso Y. Beta-relaxation of insulin molecule in lyophilized formulations containing trehalose or dextran as a determinant of chemical reactivity. *Pharm Res* 2006; 23: 961–6.
56. Yoshioka S, Miyazaki T, Aso Y. Significance of local mobility in aggregation of beta-galactosidase lyophilized with trehalose, sucrose or stachyose. *Pharm Res* 2007; 24: 1660–7.
57. Yu L, Milton N, Groleau EG, Mishra DS, Vansickle RE. Existence of a mannitol hydrate during freeze-drying and practical implications. *J Pharm Sci* 1999; 88:196–8.
58. Carpenter JF, Pikal MJ, Chang BS, Randolph TW. Rational design of stable lyophilized protein formulations: some practical advice. *Pharm Res* 1997; 14: 969–75.

14 | Stress testing of therapeutic monoclonal antibodies

Michael R. DeFelippis, Bryan J. Harmon, Lihua Huang, and Muppalla Sukumar

INTRODUCTION

Over the past 30 years, tremendous progress in the field of biotechnology has produced over a hundred protein- and peptide-based pharmaceutical products approved for a broad spectrum of diseases (1). Current industry trends for molecules entering clinical development reflect the growing importance of biopharmaceuticals, and an increasing number of these therapeutic agents will likely be commercially available in the near future (2). The growth in biopharmaceuticals is being fueled by advances in recombinant DNA and cell culture technologies (3–6) that enable large-scale and cost-effective production of exceedingly complex proteins including those containing post-translational modifications (7) that are often critical for their biological activity. Among the various classes of biotechnology-derived molecules being considered for therapeutic applications, monoclonal antibodies (mAbs) are currently receiving a great deal of attention from the pharmaceutical industry (1,8–14). The attractiveness of mAbs is largely based on their exquisite specificity coupled with their potential to stimulate effector function-mediated immune responses (15) making them ideal candidates for targeted therapy of a variety of unmet medical needs. To date, there are over 20 mAbs approved worldwide for the treatment of a variety of cancers, autoimmune, and inflammatory disorders as well as for diagnostic purposes with hundreds more in various stages of clinical development (14,16–18). Advances in protein engineering and genetic engineering technologies have now made it possible not only to develop monoclonal antibody molecules with the requisite specificity and enhanced efficacy, but also to humanize their amino acid sequence to decrease immunogenic potential (19–22).

Translating these molecules with such enormous therapeutic potential into viable commercial pharmaceuticals with requisite shelf-life stability, however, requires a careful assessment of their degradation pathways and development of effective strategies to minimize potential chemical degradation and physical denaturation (17,23,24). As with traditional, low-molecular weight pharmaceuticals, stress testing is an essential component of development programs for biotechnology-derived products. However, unlike traditional pharmaceuticals, the unique physicochemical properties of proteins and peptides impart a level of structural complexity not usually encountered with low-molecular weight molecules. In order for a biopharmaceutical product to maintain its therapeutic effect, the chemical integrity (the primary amino acid sequence and associated post-translational modifications) and unique higher order structure (the secondary, tertiary, and quaternary structure) of the active protein must be properly maintained. Disruption of the structure can potentially lead to diminished activity or have serious toxicological, immunological, or pharmacological consequences (23–26). The factors involved in manufacturing operations as well as storage, distribution, and handling of biopharmaceuticals, in general, can all potentially cause exposure to conditions that may result in perturbations to native structure (27,28). Disruption of higher order structure also has the potential to increase susceptibility to chemical degradation (27,29,30). Therefore, much of the time and effort invested in developing biopharmaceuticals involves understanding what factors affect all levels of native state structure (29–32). Stress testing plays an important role in the elucidation of relevant degradation pathways and in helping to devise corrective strategies to mitigate potential risks associated with chemical and physical instability.

This chapter deals with the subject of stress testing of biopharmaceuticals. However, it would be impossible to cover such a broad topic in a single book chapter, and we have chosen to illustrate general considerations and concepts by concentrating on mAbs for two reasons. First, the immense interest in mAbs as therapeutic agents provides an opportunity to focus

information related to the subject of stress testing on a particularly relevant representative class of molecules. Indeed, current industry and regulatory thinking on quality by design (QbD) as it relates to the development of biopharmaceuticals are directed toward potential application of these principles to therapeutic mAbs (33,34). We include in this chapter a brief description of QbD principles and how these approaches relate to stress testing of mAbs. Secondly, the structural properties of mAbs are exceedingly complex, containing hundreds of amino acids, higher order structural motifs, and post-translation modifications. Every important consideration related to stress testing of biopharmaceuticals in general can be comprehensively addressed by focusing on mAbs. Despite this concentration on mAbs, it is important to note that most, if not all, of the information provided in this chapter is generally applicable to other biopharmaceuticals such as synthetic peptides and proteins produced from cell culture with or without post-translational modification.

ANTIBODY STRUCTURE AND DEGRADATION MECHANISMS

Structural Properties of Monoclonal Antibodies

There are five classes of human secreted immunoglobulins designated as IgA, IgD, IgE, IgG, and IgM. Most therapeutic monoclonal antibodies licensed or in clinical development belong to the immunoglobulin G (IgG) class. IgG is a covalent hetero-tetramer consisting of two heavy and two light chains that are linked together through disulfide bonds. Based on primary structure, there are four subclasses (isotypes) of IgG designated: IgG1, IgG2, IgG3, and IgG4. To date, there are no IgG3 mAbs that are licensed or under clinical development. Figure 1 depicts the structures of IgG1, IgG2, and IgG4. It should be noted that there are three different structural isoforms of IgG2, i.e., IgG2-A, IgG2-B, and IgG2-A/B, which differ by the disulfide connectivity at the hinge region (35). Proteolytic cleavage of the IgG with papain produces two Fab fragments containing the antigen binding domains, and an Fc fragment which interacts with FcRn, a receptor important for controlling antibody half-life, and other receptors (e.g., Fcγs) and complexes (C1q) associated with the immune system. Each light (L) chain consists of one variable region (V_L) and a constant region (C_L) and each heavy (H) chain contains one variable (V_H) and three constant (C_H1 , C_H2 , C_H3) regions. Each variable region contains three short hypervariable sequence segments, and the amino acid sequences located here determine the antibody binding specificity. The three hypervariable segments are referred to as the complementarity-determining regions (CDRs). In contrast to the CDRs, the one constant region of each light chain and three constant regions of each heavy chain are composed of highly conserved amino acid sequences. The sequence of these constant regions is characteristic of the antibody isotypes.

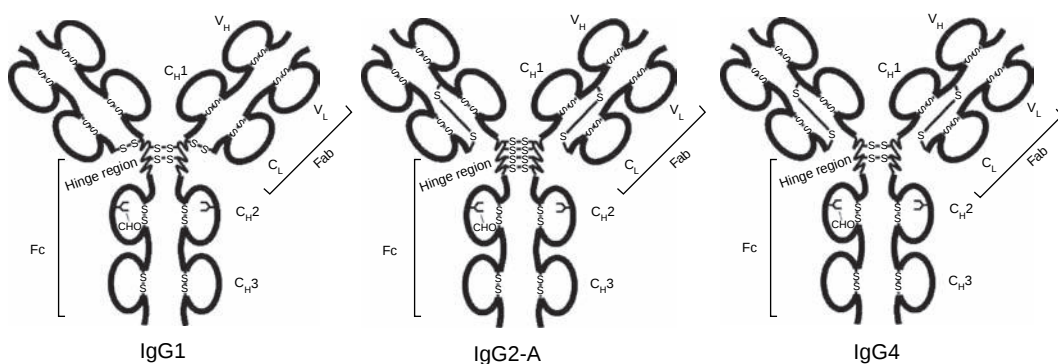


Figure 1 Schematic diagrams of human IgG1, IgG2, and IgG4 molecules. CHO indicates “carbohydrate” (i.e., consensus *N*-linked glycosylation site in C_H2 region of the heavy chain).

Each heavy chain has one consensus *N*-linked glycosylation site in the C_H2 constant region of the Fc. The oligosaccharide structures observed at this site are highly dependent upon the cell line utilized to produce the recombinant mAb (36–38). For mAbs expressed in Chinese hamster ovary (CHO) cell lines, the most common production systems utilized in the biopharmaceutical industry, these oligosaccharides are typically fucosylated, complex biantennary glycans varying in the degree of terminal β -galactosylation. Very low levels of sialylation, predominantly in the form of *N*-acetylneuraminic acid, are generally observed. mAbs that are expressed in mouse myeloma cell lines that are also commonly used in the industry, such as SP2/0 or NS0, typically demonstrate higher levels of sialylation of their oligosaccharides in the form of *N*-glycolylneuraminic acid. Approximately 20% of human serum IgGs also have the *N*-glycosylation motif Asn-X-The/Ser in the variable (Fv) region (39). For therapeutic mAbs containing glycosylation sites in the Fv region, higher levels of sialylation are typically observed for these oligosaccharides when compared to the glycans at the consensus *N*-linked glycosylation site in the C_H2 constant region of the Fc even when the mAb is expressed in CHO cell lines (40).

Degradation and Denaturation Mechanisms

The subject of protein and peptide degradation has been extensively studied, and it is well established in the scientific literature that exposure to stress conditions results in degradation of native structure via chemical and/or physical pathways (23,24,27,41). Specific amino acid side chains and certain sequences are highly susceptible to chemical modification and represent so-called hot spots in the primary structure that must be carefully managed during the development of biopharmaceuticals. A detailed description of protein and peptide degradation reactions, the associated mechanistic aspects and resulting structural characteristics of the end products is beyond the scope of this chapter. Numerous comprehensive reviews on this subject are available (23,24,27–32,41), and the reader is referred to these and the references therein for more detailed information. However, in order to provide a relevant context for explaining general considerations in the design of stress testing studies for biotechnology products, the subject is briefly covered. While stress testing studies are designed to intentionally expose molecules to atypical and extreme factors, it is important to emphasize that due to the inherent sensitivity of their primary and higher order structure, even relatively mild conditions can lead to protein and peptide degradation.

Chemical Degradation

Reactions categorized as chemical involve covalent alteration of specific amino acids or sequences of residues resulting in cleavages (hydrolysis), eliminations, additions, bond rearrangement, or cross-linking (27,41). Table 1 summarizes the types of modifications commonly resulting from exposures to pH, ionic strength, temperature, oxidizing environments, light, or other reactive species (e.g., metal ions). Many of the factors affecting degradation are explored in stress testing studies with pH, temperature, and light being routinely included because of the relevance to pharmaceutical development and the expected outcomes of deamidation, oxidation, disulfide bond chemistry, fragmentation, and cross-linking resulting from exposure to these conditions. The end products of these chemical reactions are of particular concern due to their potential to affect toxicological, biological, immunological, and/or pharmacological properties (23–27,41–43). Figure 2 illustrates the degradation pathways for several common chemical degradation mechanisms of amino acid residues. Deamidation involving loss of the α -amino groups of asparagine and glutamine residues is a common modification resulting from exposure to either acidic or alkaline pH extremes, and the reaction can be accelerated by buffer type, ionic strength, and temperature. Isomerization, racemization, or β -elimination can be likewise facilitated by extreme pH and temperature conditions. Exposure of proteins and peptides to

Table 1 Common Physicochemical Degradation Routes for Proteins and Peptides

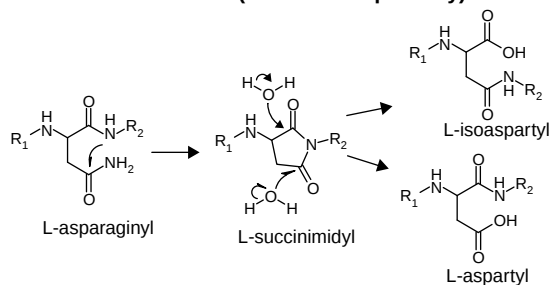
Type of Degradation	Amino Acids Residues or Sequences	Factors Affecting Degradation
Oxidation	Met, Cys, His, Trp, Tyr	Temperature, Peroxides, Light, Metal ions
Hydrolysis/fragmentation	Asp-Pro, Asp-Gly, Asp-Ser	pH, Metal ions
Deamidation	Asn, Gln	pH, Buffer species, Ionic strength, Temperature
Isomerization	Asn, Asp	pH, Buffer species, Ionic strength, Temperature
Racemization	His, Asp, Ser	pH (basic)
β -elimination	Cys, Ser, Thr, Phe, Lys	pH (basic)
Disulfide exchange	Cys-Cys	pH, Buffer species, Metal ions, Temperature, Thiols, Oxidizing agents
Desialylation	Terminal sialic acid residues on oligosaccharides	pH, Temperature
Cross-linking	Lys, Ser-Asp, Asn, Gln, Cys, Tyr	pH, Buffer species, Light, Temperature
Aggregation (includes covalent or noncovalent types) Subvisible and visible particles	No specific predictive residues (hydrophobic or charged residues may be involved)	Mechanical stress: agitation, exposure to interfaces, Hydrophobic surfaces, Temperature: heating, freezing, thawing, pH, Buffer species, Ionic strength, Metal ions, Excipients

light induces photodegradation with the peptide backbone, tryptophan, tyrosine, phenylalanine, and cysteine being primary targets (44). Certain amino acids may be oxidized by reaction with sanitizing reagents and chemical agents such as trace metal ions derived from impurities in raw materials (e.g., peroxides or aldehydes) or those leached from contact surfaces. Cysteine residues involved in disulfide bonds can undergo disulfide scrambling in a reaction facilitated by catalytic amounts of thiol. Cross-linking via specific amino acids or sequences of two contiguous residues can result from exposure to extremes of pH, temperature, or light exposure. Sequences containing Asp-X, where X is the amino acid proline, glycine, or serine, are prone to fragmentation under certain pH conditions. Furthermore, cleavage in the hinge region of IgG1 antibodies can be induced by copper ions via a nonenzymatic reaction pathway. Provided that the stress conditions are not too extreme (i.e., promote gross disruption of higher order structure leading to aggregation/precipitation), the kinetics of chemical modifications can be satisfactorily modeled using the Arrhenius relationship to allow prediction of rates for these reactions under normal storage conditions (23,24,27).

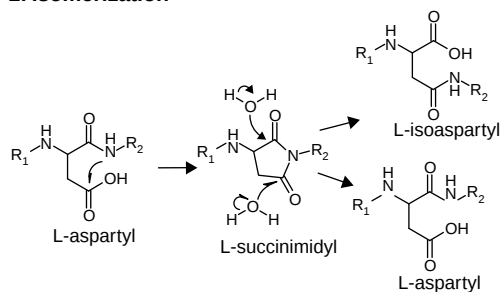
For mAbs containing very low levels of sialylation, the fucosylated, complex biantennary glycans with or without terminal β -galactosylation are relatively stable and, thus, stability of the oligosaccharide structures is typically not a major consideration during stress stability studies. However, for mAbs containing significant levels of sialylation, degradation of the oligosaccharide structures should be considered during stress stability studies due to the lability of the sialic acid linkage.

In addition to expected chemical mechanisms, degradation may be observed during stress stability testing resulting from the presence of residual enzymes not fully removed by the purification process. The most common reactions are cleavages in the peptide sequence resulting from trace levels of residual proteases (27).

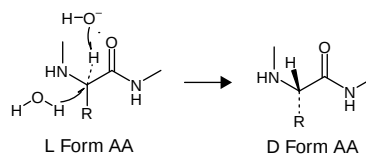
1. Deamidation of Asn (succinimide pathway)



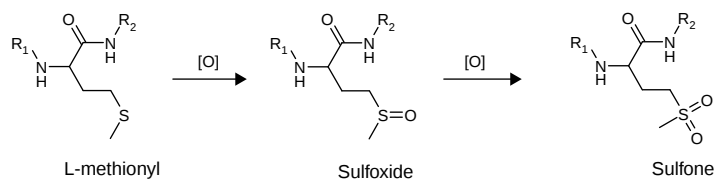
2. Isomerization



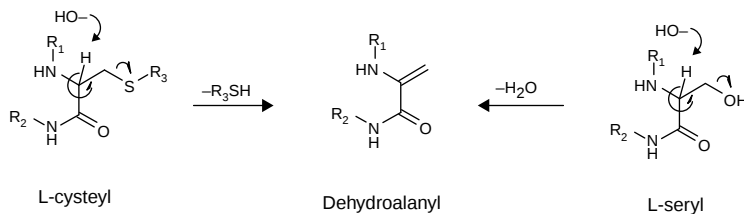
3. Racemization



4. Oxidation of met



5. β -elimination



6. Disulfide bond scrambling

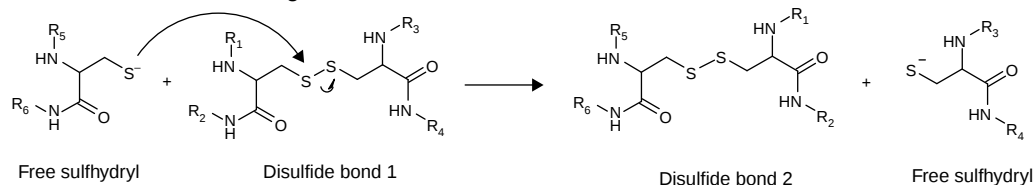


Figure 2 Common chemical degradation pathways of amino acid residues.

Physical Denaturation

Physical denaturation is unique to protein and certain peptide biopharmaceuticals and refers to destabilization of the higher order structural elements of the native conformation. Factors such as pH, ionic strength, concentration, temperature, agitation, pressure, and interfacial interactions all have the potential to cause perturbations in critical folding features of the molecule. While computer algorithms are available (45,46), predicting what specific amino acids in a peptide or protein will render the molecule susceptible to physical degradation is challenging and usually multiple residues are implicated.

In the context of stress testing, it is important to pay particular attention to the effect of temperature on higher order structure (31). As the temperature is increased, proteins unfold cooperatively at a characteristic temperature (T_m), resulting in most cases in irreversible denaturation and aggregation at high temperatures (31,32). The dominant types of chemical degradation pathways and their rates will likely be strongly influenced by temperature in the region near T_m (29,31,47,48). Therefore, the temperature for stress testing is typically chosen to be at least 10–20° lower than the onset temperature of the unfolding event to ensure that the results of stress testing are not affected by the presence of unfolded species and are relevant to the stability under real storage conditions (typically 5–25°C). In deciding an appropriate temperature for stress testing, it is important to keep in mind that some proteins may be characterized by local unfolding events at a temperature lower than the main cooperative unfolding event.

Solution pH is another important factor that can affect higher order structure and in turn the types of physical and chemical degradation and their rates. Extremes of pH can unfold proteins (typically less than pH 3.5 or greater than pH 9 for mAbs), and to the extent the molecule gets exposed to such conditions during purification or formulation process, the impact of such conditions on higher order structure needs to be assessed. Conditions that promote unfolding should be avoided unless it can be demonstrated that exposure to such conditions do not irreversibly affect critical quality attributes.

One of the degradation pathways that is linked to perturbation of higher order structure of proteins is aggregation, which can negatively affect bioactivity, bioavailability, and pharmacokinetics, or can cause immunogenicity (25–27,32,42,43,49). Protein aggregates can result from the formation of chemical cross-links between monomer molecules or simply by noncovalent association of partially unfolded molecules, because such unfolding exposes functional groups that are capable of participating in intermolecular interactions. Protein unfolding can occur due to stresses other than temperature and pH noted above; for example, as a result of exposure to air–water, ice–water, or other interfaces (50–53). As aggregation can occur from a variety of mechanisms, identifying the source of aggregation, understanding the mechanisms, and developing appropriate strategies to control aggregation is a major challenge in developing protein pharmaceuticals (32). This is further complicated by the heterogeneous nature of aggregates which makes their detection and quantitation a significant analytical challenge. Aggregates can vary enormously in their size, from small oligomers to very large aggregates potentially containing thousands of monomer units. In extreme cases, aggregation can lead to fibrillation, gelation, or subvisible/visible particles.

In considering the potential impact of higher order structural changes in the design of stress studies, an important distinction needs to be made between small peptides (~20–30 residues) and globular proteins. Unlike globular proteins, small peptides are not characterized by a unique cooperatively folded three-dimensional structure but, rather, exist as a collection of rapidly interconverting conformations. Therefore, peptides are not characterized by a cooperative unfolding transition when temperature is increased. Thus, in principle it is possible to perform stress testing on peptides at elevated temperatures (60–70°C) for faster screening of solution conditions and excipients. However, it is important to keep in mind that even in the absence of a cooperative unfolding transition, the ensemble of conformations can change as a function of temperature. Therefore, it is prudent to ensure that the Arrhenius relationship holds

across the entire temperature range so that reliable extrapolation could be made to relevant storage temperature conditions.

The relationship between chemical and physical denaturation mechanisms further complicates stress testing studies. Chemical modifications of amino acids can destabilize native-state interactions causing unfolding that ultimately leads to aggregate formation. Hydrolysis of multiple amino acid residues can produce a fragment that itself aggregates or results in exposure of hydrophobic amino acids in the remainder of the molecule that promote aggregation. Conversely, certain chemical reactions may only occur when the protein or peptide is physically denatured. It is important to note some limitations of the predictive value of stress testing, particularly as it relates to physical instability, such as irreversible aggregation and insoluble particulate formation. Unlike chemical pathways of instability, physical denaturation may be characterized by significantly non-Arrhenius (nonlinear) kinetics, making it difficult to reliably predict degradation rates based on accelerated stability studies (48,54). Without a mechanistic understanding of the physical instability, it may not be possible to adequately model such kinetics to make reliable extrapolations of data at elevated temperatures to derive the rate of degradation at actual storage temperature.

Analytical Techniques

The molecular properties of individual amino acids and overall structural features of protein and peptide biopharmaceuticals create the possibility for a diversity of potential chemical and physical reactions resulting from exposure to stress conditions (55). Except for the simplest of peptides containing only a few amino acids and lacking higher order structure, no single analytical technique can be utilized in stress testing studies to fully characterize the impact on physicochemical properties. Indeed, multiple techniques and orthogonal methods are necessary to evaluate the properties of a therapeutic monoclonal antibody having over one thousand amino acids and complex secondary and tertiary structure. ICH Q6B (56) provides a good general framework for selecting appropriate analytical tools for characterization of biopharmaceuticals.

One analytical method unique to biopharmaceuticals and particularly useful for evaluating the impact of stress conditions on biological activity is the bioassay. These complex assays are designed to measure a specific biological response relevant to the mechanism of action of the molecule (57). While all other analytical techniques reveal structural details, only the bioassay can infer that the proper conformational state of the molecule is intact and capable of functioning as intended. Technologies such as enzyme-linked immunosorbent assay (ELISA) and surface plasmon resonance (58) can also provide quantitative information on whether the chemical or physical modifications resulting from stress conditions have affected the binding affinity or binding capacity of the mAb for its target antigen (59).

Measurement Techniques for Chemical Degradation

Characterization of the chemical degradation of a mAb requires multiple complementary techniques. Fragmentation resulting from hydrolysis of peptide bonds or β -elimination of disulfide bonds is commonly profiled and quantitated by electrophoretic techniques, including sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and capillary electrophoresis in the presence of sodium dodecyl sulfate (CE-SDS) (60). While nonreducing SDS-PAGE or CE-SDS can be utilized to quantitate fragmentation, SDS-PAGE or CE-SDS performed in the presence of a reducing agent (e.g., dithiothreitol or β -mercaptoethanol) can help to characterize the integrity of the component light and heavy chains. Comparison of profiles obtained by nonreducing and reducing SDS-PAGE or CE-SDS is commonly utilized to distinguish whether observed fragments include disulfide bonds. While SDS-PAGE and CE-SDS are routinely used to quantitate fragmentation, mass spectrometry is most commonly

employed to identify the specific site of fragmentation within the peptide sequence of the light or heavy chain.

mAbs typically display charge variants (i.e., acidic and basic variants) resulting from both the inherent variability in post-translational modifications resulting from their production in biological systems as well as chemical degradation (61). Common sources of acidic variants include deamidation of asparagines or glutamine residues, pyroglutamate formation at *N*-terminal glutamine or glutamic acid residues on the heavy or light chain, glycation of the *N*-terminus or lysine residues of the heavy or light chain, sialylation of the oligosaccharide structures, and citrylation (if a citric acid buffer is utilized to formulate the mAb), while the most common source of basic variants is the presence of C-terminal lysine on the heavy chain. Depending upon the site of modification, acidic or basic variants may also be formed from fragmentation of the peptide sequence or from incorrect disulfide formation. Common methodologies for characterizing and quantitating changes in charge heterogeneity include ion-exchange chromatography (62–65), gel or capillary isoelectric focusing (60,63,66), and capillary electrophoresis (66).

While the charge heterogeneity methodologies described above are useful for observing changes in chemical structure during stress stability studies, there are several modifications that typically cannot be observed by these techniques, such as oxidation of methionine, cysteine, histidine, tryptophan, or tyrosine residues, isomerization of aspartic acid or glutamic acid residues, and racemization of histidine or aspartic acid residues. Furthermore, due to the presence of multiple types and sites of modification within the mAb and the limited resolution capabilities of the charge separation techniques, the peaks or bands observed in these analyses are often heterogeneous mixtures of molecular variants. As a result, peptide mapping (67) using proteolytic enzymes such as trypsin or Lys-C followed by reversed-phase high-performance liquid chromatography (RP-HPLC) separation of the resultant peptides generally plays a critical role in stress stability studies to more fully characterize chemical degradation. In establishing a peptide mapping method, high-resolution mass spectrometric detection (68) is commonly utilized to identify modifications based upon observed mass differences with mass spectrometric sequencing methodologies, such as collision-induced dissociation, electron capture dissociation, or electron transfer dissociation, employed to identify the specific modified residue(s) within the peptide (69,70). Due to the large number of modifications that may be present on a mAb, it is challenging to chromatographically resolve each peptide and its modified form(s) in order to quantify all modifications by UV detection. As a result, the mass spectrometric signal from LC-MS peptide mapping is also commonly used to quantify modifications. However, it should be noted that, because the presence of a chemical modification or the presence of co-eluting peptides may affect the ionization efficiency of a peptide, the results obtained by LC-MS peptide mapping are generally considered to be semi-quantitative.

Numerous methods have been developed to profile the glycans associated with mAbs. The most commonly used methodologies utilize the enzymatic release of the glycans by PNGase F, labeling of the glycans with a fluorophore, and separation of the labeled glycans by either liquid chromatography (71,72) or capillary electrophoresis with fluorometric detection (73).

Measurement Techniques for Physical Denaturation

Thermal unfolding of proteins can be monitored either by calorimetric methods, such as differential scanning calorimetry (74), or spectroscopic methods, such as circular dichroism (CD) and fluorescence spectroscopy (75–78). Briefly, far and near-UV CD spectroscopy provides information on the secondary and tertiary structure of proteins, respectively, while fluorescence spectroscopy can be used to specifically probe the micro-environment of tryptophan residues within the protein and to obtain information on protein dynamics. Fourier-transform infrared (FT-IR) also provides information on the secondary structure of

proteins (79,80) but is more sensitive to beta-sheet structure, compared to far-UV CD, and can be readily used on different sample types, such as solutions, solids, and gels. Recent developments in multidimensional nuclear magnetic resonance (NMR) now make it possible to obtain fairly detailed structures of peptides and small proteins (10–30 kDa) in solution (81,82). However, structure determination by NMR even for small proteins requires considerable effort and labeling with ^{13}C and ^{15}N isotopes. Therefore, although the higher order structures of many pharmaceutically important peptides and proteins have been characterized by NMR (81,82), it is not routinely used in the pharmaceutical industry. Large proteins, such as mAbs, are currently outside the reach of structural determination by NMR. Therefore, while CD, fluorescence, and FT-IR spectroscopic methods do not provide the level of structural detail obtainable by NMR, they are relatively easier to use and interpret, and lend themselves to higher throughput screening of the impact of solution conditions on higher order structure and, therefore, are more commonly used in the pharmaceutical industry.

Because of the heterogeneity and the broad size range of aggregates, no single technique is ideal for measuring all types of aggregates. As a result, a variety of orthogonal techniques are often necessary for comprehensive detection and control of aggregates in pharmaceutical samples, e.g., size exclusion chromatography (SEC), analytical ultracentrifugation (AUC), dynamic light scattering (DLS), and asymmetric flow field flow fractionation (AF4) for soluble aggregates, and light obscuration, turbidimetry, and microscopy techniques for subvisible and visible particulates (83–88). Due to the capability of SDS to dissociate noncovalent interactions, SDS-PAGE or CE-SDS can provide information on the presence of aggregate resulting from covalent cross-linking or strong noncovalent interactions. Comparison of profiles obtained by nonreducing and reducing SDS-PAGE or CE-SDS is commonly utilized to determine whether covalent aggregation involves disulfide cross-linking.

STRESS TESTING AS A PRODUCT DEVELOPMENT TOOL

ICH Guidelines

Stress testing is an essential subset of the studies associated with biopharmaceutical product development stability programs, and the available guidance set forth in ICH Q1A(R2) “Stability Testing of New Drug Substances and Products” is generally applicable (89). However, the unique molecular characteristics of proteins and peptides have been taken into consideration by ICH resulting in publication of the Q5C “Stability Testing of Biotechnology / Biological Products” guideline that provides specific information related to this class of pharmaceuticals (90). The scope of ICH Q5C includes monoclonal antibodies. Both guidance documents address the subject of stress testing albeit in somewhat limited detail.

ICH Q1A(R2) provides the basic rationale and framework for conducting pharmaceutical stress testing emphasizing that such studies can help identify likely degradation products and pathways, define the molecule’s intrinsic stability, and validate stability indicating power of analytical methods. This guideline indicates that the nature of stress testing depends on the drug substance and type of drug product but does not include many specific details particularly for evaluation of drug products. Nevertheless, the text of ICH Q1A(R2) does indicate that stress testing is likely conducted on a single batch of drug substance and should include the effect of temperature in 10°C increments (e.g., 50°C, 60°C, etc., above accelerated conditions), humidity (e.g., 75% RH or greater) where appropriate, oxidation, photolysis, and susceptibility to hydrolysis over a wide range of pH values. Photostability is specifically mentioned as being an integral part of stress testing and reference is made to ICH Q1B “Photostability Testing of New Drug Substances and Products” (91).

ICH Q5C (90) recognizes the important relationship between molecular conformation and biological activity for proteins or peptides as the active substance and how this linkage should be incorporated into the strategies for designing stability studies in general. Maintaining the proper noncovalent and covalent structural features of these molecules is

challenging due to their extreme sensitivity to environmental factors such as temperature, oxidation, light, ionic strength, and physical perturbations (e.g., exposure to interfaces and agitation). For these reasons, it is generally understood that most extreme conditions for stress testing studies outlined in ICH Q1A(R2) (89) may not be appropriate for biotechnology/biological products, and choice of stress conditions should be made on a case-by-case basis (90). This point is particularly noteworthy given that the typical storage condition for mAb drug substances is -70°C or 5°C , while drug product solutions are routinely maintained between 2°C and 8°C . These temperature requirements result in the selection of 25°C and 40°C as the conditions generally used for accelerated and stress stability testing, respectively. While ICH Q5C (90) cites the same reasons for conducting stress testing as ICH Q1A(R2) (89), the guideline additionally points out that studies under stress conditions could provide useful information on the impact of accidental exposures outside of those proposed for the product. Such knowledge is especially relevant to defining transportation, storage, and use requirements for biotechnology/biological products, which typically require very stringent controls on temperature and handling to avoid degradation. Temperature excursions and inadvertent mishandling are very common failure modes for bioproducts. However, as discussed later in this section, stress testing for biotechnology/biological products is used more comprehensively as a product development tool with multiple studies conducted for a variety of purposes throughout the product development lifecycle.

Stress Testing as an Element of Quality by Design

Stress testing plays a critical role throughout the product lifecycle of a therapeutic monoclonal antibody, and it is essential to a “quality by design” approach to biopharmaceutical product development (92,93). Risk assessments based upon the results of stress testing studies are important components in the design and optimization of the candidate molecule as well as the drug substance and drug product matrices and storage conditions and the analytical methodologies utilized to characterize the mAb.

During the molecule design stage, stress testing of candidate molecules can identify specific molecular attributes, such as labile residues in the CDRs or a high propensity for aggregation or fragmentation, that jeopardize the development of the candidate molecule. This information can be utilized to select the most viable candidate among multiple candidates or can be used to identify the need for further optimization of a candidate molecule’s amino acid sequence in order to mitigate risk. Once a candidate molecule is brought forward into pharmaceutical development, stress testing plays an important role in identifying drug substance and drug product matrices that provide the necessary stability to support early phase toxicological and clinical studies and to assess the stability-indicating capabilities of analytical methods. Stress testing is also important in identifying special manufacturing or storage requirements resulting from specific molecular attributes, such as sensitivity to light or metal ions (e.g., iron or copper ions).

During late-phase development, stress testing is critical to inform design and optimization of the drug substance matrix and container/closure system as well as the drug product formulation and container/closure system or delivery device. Knowledge of the chemical degradation and physical denaturation mechanisms observed during these stress stability studies also provides direction for the development of appropriate analytical methods and justification for the analytical control strategy. Once the analytical methods and drug substance and drug product matrices have been finalized, formal stress stability studies, as described by ICH Q1A(R2) (89), are required to meet regulatory expectations. During late-phase clinical development and in commercial manufacturing, changes to the drug substance or drug product manufacturing processes require comparability assessments, as described in ICH Q5E (94). Stress testing studies to assess whether the proposed process change has affected the chemical degradation or physical denaturation mechanisms of the drug substance or drug product is increasingly becoming a regulatory expectation.

Risk Assessments Associated with Stress Testing

Because of the size and complexity of mAbs, exposure to stress conditions typically results in the observation of multiple chemical degradation or physical denaturation mechanisms. Furthermore, these degradation mechanisms are superimposed upon the inherent heterogeneity of the mAb resulting from post-translational modifications, due to its production in a biological system. This heterogeneity is illustrated in Figure 3, which shows the modifications observed for a therapeutic IgG4 molecule in our laboratory (unpublished data).

In light of this extensive heterogeneity, it is important to focus on the degradation mechanisms that are most relevant to the safety and efficacy of the mAb. Risk assessments can be utilized to assess the potential liabilities associated with the degradation mechanisms and to identify potential mitigation strategies. These risk assessments should be refined throughout development as additional information becomes available. Some considerations in the risk assessments may include the following:

- 1. Is the potential impact of the degradation established in the scientific literature? For example, as noted above, aggregation has been reported to have the potential to negatively affect bioactivity, bioavailability, and pharmacokinetics, or can cause immunogenicity (25–27, 32,42,43,49). While these impacts can be highly molecule specific, this literature would certainly heighten the potential risk associated with developing a mAb that is highly susceptible to aggregation.
- 2. Where is the location of the degradation? When assessing the impact of stress testing on the structural properties of therapeutic mAbs, it can be expected that degradation of amino acid residues located in the CDRs are more likely to result in reduction of antigen binding capability and subsequent loss of biological activity (59). The risk assessment may prompt additional studies to evaluate the antigen binding properties of the degraded mAb in order to better assess the impact of the degradation mechanism. In contrast, degradation within

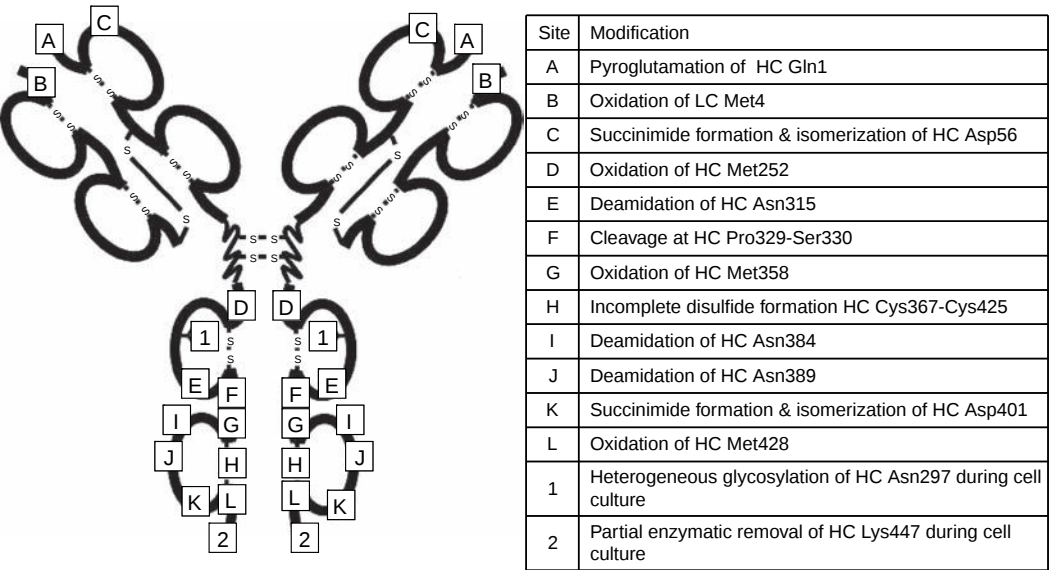


Figure 3 Post-translational modifications and chemical degradation mechanisms observed for a therapeutic IgG4 mAb. Primary degradation mechanisms observed during stress stability studies are indicated by letters. Post-translational modifications indicated by numbers are additional sources of heterogeneity resulting from cell culture process that did not change appreciably during stress stability studies (author's unpublished results). HC and LC indicate heavy chain and light chain, respectively.

the constant regions may have less impact, particularly, if the mAb is designed specifically for neutralization of a therapeutic target without requirement for effector function.

3. What is the mechanism of action of the therapeutic mAb? While modifications to the Fc fragment may be less critical for mAbs whose mechanism of action requires only binding to a target antigen, modifications to amino acids located in key regions of the Fc fragment could be critical for mAbs whose mechanism of action involves effector function. The risk assessment may trigger additional studies to evaluate the binding properties of the degraded mAb to relevant Fc receptors in order to better assess the impact of the observed degradation mechanism.
4. How effectively can the degradation mechanism be controlled by choice of the manufacturing processes, matrices, container closure systems, and storage conditions for the drug substance and drug product? Since the degradation mechanisms of mAbs can be highly dependent upon pH, temperature, exposure to oxidizing agents, light exposure, and physical stress, the stress stability studies should guide further process and formulation development and optimization in order to minimize the risk associated with critical degradation mechanisms.
5. Is the degradation likely to occur in-vivo? Due to the relatively long half-lives of therapeutic mAbs typically ranging from days to months, certain chemical degradation mechanisms that are observed during stress stability studies may be expected to also occur during administration of therapeutic mAbs. For example, deamidation has been shown to occur in biological matrices (95). If the particular degradation mechanism does not affect the safety or efficacy of the mAb, the observation that this degradation mechanism also occurs in vivo may be useful to justify that a particular degradation mechanism is not a critical quality attribute of the drug product. However, significant risk may be introduced if the degradation mechanism does affect the safety or efficacy of the therapeutic mAb and is likely to happen in vivo. Therefore, this risk assessment may prompt additional studies to determine whether or not a degradation mechanism occurs in vivo.

Stress-Testing Examples

Parameters commonly evaluated during stress stability studies include exposure to pH ranges, temperature ranges, oxidative conditions, light conditions, and physical stress. The following sections provide some considerations for performing these studies and illustrate specific examples of the findings that have been reported for mAbs during these types of studies.

pH

The pH of a mAb solution can greatly affect almost all the chemical degradation pathways listed in Table 1. pH can also affect the physical stability of mAbs as it alters the number and distribution of charges on the protein surface. Several published reports have shown that low pH can cause a loss of tertiary structure or result in precipitation during stress stability (96,97). With respect to chemical degradation, higher pH favors deamidation, racemization, hinge cleavage, disulfide bond scrambling, and β -elimination. In contrast, isomerization (succinimide formation) and Asp-Xxx cleavage (especially Asp-Pro cleavage) commonly occur at lower pH. Generally, a series of stress testing studies covering a wide range of pH (typically pH 4–9) is required to identify the optimal pH for physicochemical stability of a specific mAb.

Nonenzymatic deamidation of asparagine (Asn) or glutamine (Gln) is a major degradation pathway of peptides or proteins and can occur spontaneously during manipulation, purification, and long-term storage. Deamidation can potentially cause structurally and biologically important alterations in peptides and proteins through the introduction of unfavorable negative charge. For example, the mAb, anti-IL1 β has one potential deamidation site at Asn55 in the heavy chain CDR2. The binding activity was found to be 18 times lower when Asn55 was replaced with Asp55 to simulate the impact of deamidation at this site (95). In other studies (98),

the stability of this mAb was explored in the pH 4.0–7.0 range, and the results demonstrated that the rates of single and double site deamidation at 37°C are five to six times faster at pH 7, compared to those at pH 5.

In addition to primary sequence, the higher order structural context can greatly influence the rates of chemical degradation, for example, Wakankar et al. (99) studied aspartate isomerization in the CDRs of two similar mAbs and pentapeptide models of the mAbs. The isomerization rate of one mAb was less pH dependent and faster than that of the model peptide for pH > 6 while the isomerization rate of the other mAb was pH dependent as it decreased when pH increased from 5 to 8, and was slower than that of the model peptide. There are two Asp-Gly sequences in the constant regions of IgG1, IgG2, and IgG4 mAbs. Aspartic acid residues in these positions can potentially be isomerized to isoaspartic acid. Our studies have shown that aspartic acid isomerization at these two positions only occurs at pH <5 (unpublished data).

Temperature

To define a formulation design space, stress stability studies at elevated temperature are usually conducted during preformulation and/or formulation development. However, estimation of room temperature or 2–8°C stability based on stress stability studies for mAbs, like other biopharmaceuticals, may or may not be possible or accurate. The limited predictability is due to the presence of complex and multiple degradation pathways, which may have different degrees of temperature dependences. For example, Kroon et al. (67) found that relative to other degradation pathways for the monoclonal antibody, OKT3 (IgG2a), Asn deamidation was preferentially accelerated with increasing temperature. On the other hand, Paborji et al. (100) investigated loss of monomer in a chimeric antibody (L6) by SEC-HPLC after exposing pH 7.2 samples to temperatures in the range of 30–50°C and found that the data followed Arrhenius behavior allowing estimation of the antibody stability at 2–8°C by using the SEC data collected at high temperatures. Therefore, caution should be exercised when interpreting data obtained from high-temperature stress studies for predictive stability purposes. Refer to section “*Physical Denaturation*” for general guidance regarding the choice of appropriate temperature for accelerated stability studies and its relationship to the unfolding temperature of a protein molecule. The exact temperature chosen for accelerated stability studies and, in turn, the length of the study will have to be determined on a case-by-case basis.

Oxidative Conditions

Oxidation can easily occur in mAbs during cell culture, purification, and long-term storage. Methionine is one of the most susceptible amino acids for oxidation in proteins. Depending on sequence location, the oxidation of amino acid residues may significantly decrease antibody binding activity for antigen (101), affect the binding of human IgG1 to FcRn and Fc gamma receptors (102), affect binding to protein A and protein G resins used for purification (103), or cause aggregation (104,105). To probe the amino acid residues susceptible to oxidation, an antibody solution (typically 1.0 mg/mL) is spiked with low levels of H₂O₂ (0.01–0.1%), *tert*-butylhydroperoxide (tBHP) (0.1–1%) or ozone (101) at ambient temperature. The hydroxyl radical rapidly oxidizes a variety of amino acid side chains: most notably, sulfur-containing residues and aromatic residues (106). As a result, H₂O₂ is often used to investigate amino acid oxidation for mAb samples (107,108). tBHP is another reagent often used for investigating amino acid oxidation of mAbs (101,109–111). Treatment with tBHP has been shown to only oxidize Met residues (109,110) on solvent exposed surfaces of the antibody. The antibody solution can also be light-irradiated (101) or stressed at elevated temperature to probe the susceptibility to amino acid oxidation.

There is no ideal analytical method for monitoring oxidation of intact mAbs although oxidation of amino acids does make the molecule more hydrophilic, and separation between oxidized and native mAbs may be achieved by chromatographic methods for specific molecules.

For example, Chumsae et al. (111) found that IgG1 antibodies with oxidized Met residues eluted slightly later using weak cation exchange chromatography. A single oxidized Trp residue in the CDR3 of IgG2 heavy chain made it possible to separate oxidized heavy chain or mAb from unoxidized heavy chain or mAb using SEC and RP-HPLC (112). After papain digestion, an impurity with oxidized Met residues in the Fc region elutes earlier relative to the unoxidized molecule on a HIC column (109,113). Despite these examples, to characterize oxidation of susceptible amino acids of mAbs, peptide mapping with online LC-MS is generally required. As expected, oxidized Met-containing peptides elute earlier on RP-HPLC and have generally 16 (for Met or Trp oxidation), 4, or 32 Da (for Trp oxidation) higher mass compared to their respective unoxidized versions.

Usami et al. (107) studied oxidation of human monoclonal antibody C23 spiked with 0.01% or 0.1% H₂O₂. The stressed samples were collected at 0, 3, 7, and 14 days after storage at two temperatures (8 and 37°C) and characterized by SEC-HPLC, hydroxyapatite (HA)-HPLC, SDS-PAGE, isoelectric focusing (IEF), and MALDI-TOF MS. Under the stress conditions, the monomer content of mAb C23 decreased and approximately followed first-order reaction kinetics. In other work, the oxidation of two similar IgG1 mAbs, MEDI-493 and MEDI-524, was studied after the native molecules were treated with 1% tBHP or different amounts of ozone or exposure to UV light for various times (101). The stress samples were characterized by F protein binding ELISA and online LC/MS/MS. The major oxidation sites were identified at Met101, Met255, Met431, and Trp105 of the heavy chain, and the reaction rates followed first-order kinetics for UV light exposure and tBHP treatment. These results suggested that the susceptible residues were located on the solvent-accessible surface of the native protein. Comparing the different stress conditions, the authors showed that oxidation of the Trp105 located in the heavy chain CDR3 of MEDI-493 resulted in the greatest activity loss.

Light Exposure

Proteins undergo a variety of chemical reactions when exposed to near-ultraviolet and visible light (44). While the aromatic residues (Trp, Tyr, and Phe) and cysteine are the primary targets of light-induced photo-oxidation, reactive oxygen species generated in the process can cause indirect oxidation with histidine residues being most susceptible, particularly, in the presence of contaminating metal ions. Exposure to visible and UV light can potentially occur during purification (e.g., glass columns), UV detection during chromatographic operations, fill/finish operations, visual inspection, and during delivery (e.g., clear IV bags). Primary degradation products include oxidation products of tryptophan, such as kynurenine, and dityrosine formation from tyrosine that can result in the formation of dimer and higher order aggregates via intermolecular covalent cross-links. Both kynurenine and dityrosine can be easily monitored by their unique fluorescence spectra. Formation of cystine radical anion, either by direct capture of an electron or indirectly via Trp and Tyr, leads to thiyl radical that can potentially form intra or intermolecular disulfide cross-links.

ICH Q1B (91) recommends that photostability testing is carried out with light sources with an output that mimics outdoor (D65 emission standard) or indoor (ID65 emission standard) daylight or, alternately, by exposure to both cool white fluorescent light (400–700 nm) and near UV (320–400 nm) fluorescent light. The guidance also specifies an exposure level of 1.2 million lux hours and integrated near-UV energy of 200 watt hours/m² in confirmatory studies to demonstrate that the drug substance and drug product are photostable under conditions of handling, packaging, and labeling. Forced degradation studies intended to evaluate the overall photosensitivity and to facilitate method development and degradation pathway elucidation can use a variety of exposure conditions depending on the photosensitivity of the molecule. However, light exposure within ICH guidelines is typically employed for mAbs. Absorption of light by susceptible residues can result in multiple competing pathways of chemical reactions (see Kerwin and Remmele (44) for a comprehensive review), and the relative composition of the degradation products depend on factors like temperature, presence of

molecular oxygen, and trace metals. For example, generation of electrons during Trp or Phe photoreaction, relative to other competing pathways, is highly dependent on temperature with higher temperatures strongly favoring electron generation whereas similar reactions from Tyr are less temperature dependent. Other factors related to higher order structure include solvent exposure of the reactive groups and spatial proximity of Trp, Tyr, Phe, and cystine residues that can potentially be affected by solution conditions such as pH and ionic strength. For these reasons, the temperature, pH, and ionic strength should be carefully chosen to mimic likely exposure conditions. Given the sensitivity of photo-degradation pathways to temperature, precautions should be taken to minimize local heating during light exposure.

Physical Stress

Interfacial and Mechanical Stresses

Since one of the goals of stress studies is to understand the potential pathways of degradation, both chemical and physical, and identify potential means to measure and minimize such pathways, in designing stress studies it is important to consider the likely stresses the molecule will experience during the API and drug product manufacturing process, storage, shipping, and administration. Proteins are surface active due to their amphiphilic nature and, therefore, have a tendency to accumulate at interfaces (50–53), e.g., air–water, ice–water, membrane–water, or other interfaces. Due to the interfacial nature of such environments, the accumulated fraction can undergo perturbation of higher order structure that can in turn lead to aggregation (50). This type of surface-induced protein aggregation can occur during mixing, pumping, filling, spray drying, or shipping due to exposure to air–water or other interfaces; during freeze-thaw and freeze-drying operations due to exposure to ice–water interfaces; and during filtration operations due to exposure to membrane–water interfaces (50–53,114,115). While there is a concern that shear experienced during manufacturing operations can cause protein unfolding and aggregation, several carefully designed studies where the confounding effects of air–water interface were absent clearly showed that shear stresses well in excess of those typically experienced during manufacturing operations did not result in any detectable unfolding or aggregation (116,117). Calculations also show that the forces experienced during manufacturing due to shear (<0.06 pN) are small compared to those expected at the air–water interface (140 pN) or those required to mechanically unfold proteins (20–150 pN) (117).

The susceptibility of proteins to mechanical and interfacial stress they experience during manufacturing operations, storage, and shipping can be mimicked in agitation studies using horizontal or vertical shakers, in stirring studies in a vial, or in pumping studies (86,87,117–120). Compared to thermal stability testing, such studies are carried out for relatively short duration, typically 24–48 hours. Designing these studies with and without varying levels of a surfactant will help determine if the observed degradation (aggregation, particulate formation) is a result of exposure to an interface and will also help identify appropriate levels of the surfactant that would confer a protective effect. In such studies, the impact of air–water interface or other interfaces could also be delineated by conducting stirring studies with or without head-space. In a recent study using an IgG1 antibody (87), it was noted that the type and extent of aggregates generated were dependent on the way in which agitation was performed as well as the temperature and duration of the stress study. Stirring resulted in formation of insoluble visible and subvisible particles, presumably due to interaction with glass and/or Teflon surfaces, while shaking induced higher levels of soluble aggregates, likely due to interactions at the air–water interface. Therefore, the parameters of an agitation study need to be carefully chosen to appropriately mimic expected stress.

Some recent studies have highlighted the potential for nucleation and growth of protein aggregates and particulates as a result of interaction with micro- and nanoparticles shed by protein contact surfaces during manufacturing operations, such as by pumps, tubing, and filters, or those shed by container closures (e.g., glass, rubber, and silicone) (121–123). To rapidly assess the susceptibility of proteins to these types of physical degradations, stress

study protocols that utilize glass, cellulose, stainless steel, and iron(III) oxide microparticles have been proposed (121).

Freeze-thaw

Freezing and thawing operations expose the protein to ice–water interfaces in addition to low temperatures that are potentially destabilizing (31,51,53,114). Some of the factors to consider in designing experiments to evaluate freeze-thaw stress are the rate of freezing, protein concentration, and container geometry (114,124). The area of the ice–water interface is a function of the ice crystal size, which in turn is related to freezing rate with faster cooling rates resulting in smaller ice crystals and, hence, higher surface area (114). In addition to influencing the crystal size, freezing rate also affects the concentration gradients with slower freezing rates generally resulting in more significant concentration gradients. In general, higher protein concentrations are more resistant to surface-induced aggregation (125). While this trend may be counter-intuitive, it is consistent with the expectation that a finite area of the ice–water interface can damage only a certain number of molecules which constitute a smaller fraction at higher protein concentration (126). As with agitation-induced stress, freeze-thaw induced damage can be typically controlled by adding either a surfactant, e.g., polysorbate, or a preferentially excluded excipient (51).

While the mechanical stress studies described above allow qualitative assessments of susceptibility of a given protein to agitation and other interfacial stresses and rank ordering of various formulation options in terms of their protective effect, it is not possible to make quantitative prediction of actual degradation rates under typical handling conditions. In situations where the rate-limiting step in the formation of proteinaceous particles involves the formation of a critical aggregated nucleus, the appearance of insoluble particles can be stochastic, further making it challenging to extrapolate findings from a short-term stability study. Thus, fairly lengthy studies at representative storage conditions may be necessary to identify formulations with optimal physical stability or confirm the findings from an accelerated stress study.

CONCLUSIONS

The unique molecular properties of mAbs make them attractive therapeutic agents for a variety of medical applications. Much time and effort is focused throughout discovery and product development to optimize physicochemical features to increase stability and enhance pharmacological attributes. Typical of most protein biopharmaceuticals, the structure–function relationships associated with mAbs necessitate a thorough understanding of the amino acid residues and higher order structural motifs important to quality, safety, and efficacy. Stress testing plays a significant role along with other studies to generate the knowledge required to produce mAbs having the requisite stability and capable of performing consistently in pharmaceutical applications.

REFERENCES

1. Aggarwal S. What's fueling the biotech engine? *Nat Biotechnol* 2007; 25: 1097–104.
2. Walsh G. Biopharmaceutical benchmarks. *Nat Biotechnol* 2006; 24: 769–76.
3. Palomares LA, Estrada-Mondaca R, Ramirez OT. Production of recombinant proteins: challenges and solutions. *Methods Mol Biol* 2004; 267: 15–52.
4. Gerngross TU. Advances in the production of human therapeutic proteins in yeasts and filamentous fungi. *Nat Biotechnol* 2004; 22: 1409–14.
5. Wurm F. Production of recombinant protein therapeutics in cultivated mammalian cells. *Nat Biotechnol* 2004; 22: 1393–8.
6. Birch JR, Racher AJ. Antibody production. *Adv Drug Del Rev* 2006; 58: 671–85.
7. Walsh G, Jefferis R. Post-translational modifications in the context of therapeutic proteins. *Nat Biotechnol* 2006; 24: 1241–52.
8. Baker M. Upping the ante on antibodies. *Nat Biotechnol* 2005; 23: 1065–72.

9. Reichert JM, Rosensweig CJ, Faden LB, et al. Monoclonal antibody success in the clinic. *Nat Biotechnol* 2005; 23: 1073–8.
10. Glennie MJ, Johnson PWM. Clinical trials of antibody therapy. *Immunol Today* 2000; 21: 403–10.
11. Reichert JM, Dewitz MC. Anti-infective monoclonal antibodies: perils and promise of development. *Nat Rev Drug Discov* 2006; 5: 191–5.
12. Reichert JM, Valge-Archer VE. Development trends for monoclonal antibody cancer therapeutics. *Nat Rev Drug Discov* 2007; 6: 349–56.
13. Marasco WA, Sui J. The growth and potential of human antiviral monoclonal antibody therapeutics. *Nat Biotechnol* 2007; 25: 1421–34.
14. Swann PG, Tolnay M, Muthukumar S, et al. Considerations for the development of therapeutic monoclonal antibodies. *Curr Opin Immunol* 2008; 20: 493–9.
15. Salfeld JG. Isotype selection in antibody engineering. *Nat Biotechnol* 2007; 25: 1369–72.
16. Weinberg WC, Frazier-Jessen MR, Wu WJ, et al. Development and regulation of monoclonal antibody products: challenges and opportunities. *Cancer Metastasis Rev* 2005; 24: 569–84.
17. Daugherty AL, Mrsny RJ. Formulation and delivery issues for monoclonal antibody therapeutics. *Adv Drug Del Rev* 2006; 58(5–6): 686–706.
18. Parren PWHI, van de Winkel JGJ. An integrated science-based approach to drug development. *Curr Opin Immunol* 2008; 20: 426–30.
19. Lonberg N. Fully human antibodies from transgenic mouse and phage display platforms. *Curr Opin Immunol* 2008; 20: 450–9.
20. Presta LG. Molecular engineering and design of therapeutic antibodies. *Curr Opin Immunol* 2008; 20: 460–70.
21. Raju TS. Terminal sugars of Fc glycans influence antibody effector functions of IgGs. *Curr Opin Immunol* 2008; 20: 471–8.
22. Labrijn AF, Aalberse RC, Schuurman J. When binding is enough: nonactivating antibody formats. *Curr Opin Immunol* 2008; 20: 479–85.
23. Frokjaer S, Otzen DE. Protein drug stability: a formulation challenge. *Nat Rev Drug Discov* 2005; 4: 298–306.
24. Cleland JL, Powell MF, Shire SJ. The development of stable protein formulations: a close look at protein aggregation, deamidation, and oxidation. *Crit Rev Ther Drug Carrier Syst* 1993; 10: 307–77.
25. Schellekens H. Bioequivalence and the immunogenicity of biopharmaceuticals 2002. *Nat Rev Drug Discov* 2002; 1: 457–62.
26. Sharma B. Immunogenicity of therapeutic proteins: Part 1. Impact of product handling. *Biotechnol Adv* 2007; 25: 310–17.
27. Ahern TJ and Manning MC Eds. Stability of Protein Pharmaceuticals Part A. Chemical and physical pathways of protein degradation. New York: Plenum Press, 1992.
28. Wang W. Protein aggregation and its inhibition in biopharmaceuticals. *Int J Pharmaceut* 2005; 289 (1–2): 1–30.
29. Meyer JD, Ho B, and Manning MC. Effects of conformation on the chemical stability of pharmaceutically relevant polypeptides. In: Carpenter JF and Manning MC eds. Rational design of stable protein formulations. Theory and practice. (Pharmaceutical Biotechnology Vol. 13). New York: Kluwer Academic/Plenum Publishers, 2002: 85–107.
30. Kamerzell TJ, Middaugh CR. The complex inter-relationships between protein flexibility and stability. *J Pharm Sci* 2008; 97: 3494–517.
31. Volkin DB and Middaugh C. The effect of temperature on protein structure. In: Ahern TJ and Manning MC eds. Stability of Protein Pharmaceuticals: Part A. Chemical and Physical Pathways of Protein Degradation. (Pharmaceutical Biotechnology Vol. 2). New York: Plenum Press, 1992: 215–47.
32. Chi EY, Krishnan S, Randolph TW, et al. Physical stability of proteins in aqueous solution: mechanism and driving forces in nonnative protein aggregation. *Pharm Res* 2003; 20: 1325–36.
33. Kozłowski S, Swann P. Current and future issues in the manufacturing and development of monoclonal antibodies. *Adv Drug Del Rev* 2006; 58: 707–22.
34. Rathore AS, Winkle H. Quality by design for biopharmaceuticals. *Nat Biotechnol* 2009; 27: 26–34.
35. Wypych J, Li M, Guo A, et al. Human IgG2 antibodies display disulfide-mediated structural isoforms. *J Biol Chem* 2008; 283: 16194–9205.
36. Raju TS, Briggs JB, Borge SM, et al. Species-specific variation in glycosylation of IgG: evidence for the species-specific sialylation and branch-specific galactosylation and importance for engineering recombinant glycoprotein therapeutics. *Glycobiology* 2000; 10: 477–86.

37. Jefferis R. Glycosylation of recombinant antibody therapeutics. *Biotechnol Prog* 2005; 21: 11–16.
38. Sheeley DM, Merrill BM, Taylor LCE. Characterization of monoclonal antibody glycosylation: comparison of expression systems and identification of terminal α -linked galactose. *Anal Biochem* 1997; 247: 102–10.
39. Abel CA, Spiegelberg HL, Grey HM. The carbohydrate contents of fragments and polypeptide chains of human gamma-G-myeloma proteins of different heavy-chain subclasses. *Biochemistry* 1968; 7: 1271–8.
40. Lim A, Reed-Bogan A, Harmon BJ. Glycosylation profiling of a therapeutic recombinant monoclonal antibody with two N-linked glycosylation sites using liquid chromatography coupled to a hybrid quadrupole time-of-flight mass spectrometer. *Anal Biochem* 2008; 375: 163–72.
41. Goolcharran C, Khosravi M, Borchardt RT. Chemical pathways of peptide and protein degradation. In: Frokjaer S, Hovgaard L, eds. *Pharmaceutical Formulation Development of Peptides and Proteins*. London: Taylor & Francis, 2000: 70–88.
42. Brorson K, Phillips J. Defining your product profile and maintaining control over it: part 4. Product-related impurities: Tackling aggregates. *Bioproc Intl* 2005; 3: 50–4.
43. Rosenberg AS. Effects of protein aggregates: an immunologic perspective. *AAPS Journal* 2006; 8: E501–E507.
44. Kerwin BA, Rummelle RL. Protect from light: photodegradation of protein biologics. *J Pharm Sci* 2007; 96: 1468–79.
45. Tartaglia GG, Pawar AP, Campioni S, et al. Prediction of aggregation-prone regions in structured proteins. *J Mol Biol* 2008; 380: 425–36.
46. Caflisch A. Computational models for the prediction of polypeptide aggregation propensity. *Curr Opin Chem Biol* 2006; 10: 437–44.
47. Remmele RL Jr, Bhat SD, Phan DH, et al. Minimization of recombinant human Flt3 ligand aggregation at the Tm plateau: a matter of thermal reversibility. *Biochemistry* 1999; 38: 5241–7.
48. Roberts CJ, Darrington RT, Whitley MB. Irreversible aggregation of recombinant bovine granulocyte-colony stimulating factor (bG-CSF) and implications for predicting protein shelf life. *J Pharm Sci* 2003; 92: 1095–111.
49. Cromwell MEM., Hilario E., Jacobson F. Protein aggregation and bioprocessing. *AAPS J* 2006; 8: E572–79.
50. Carpenter JF, Kendrick BS, Chang BS, et al. Inhibition of stress-induced aggregation of protein therapeutics. *Methods Enzymol* 1999; 309: 236–55.
51. Chang BS, Kendrick BS, Carpenter JF. Surface-induced denaturation of proteins during freezing and its inhibition by surfactants. *J Pharm Sci* 1996; 85: 1325–30.
52. Wahlgren M, Arnebrant T. Protein adsorption to solid surfaces. *Trends Biotechnol* 1991; 9: 201–8.
53. Strambini GB, Gabellieri E. Proteins in frozen solutions: evidence of ice-induced partial unfolding. *Biophys J* 1996; 70: 971–6.
54. Weiss WF 4th, Young TM, Roberts CJ. Principles, approaches, and challenges for predicting protein aggregation rates and shelf life. *J Pharm Sci* 2009; 98: 1246–77.
55. Harris RJ. Heterogeneity of recombinant antibodies: linking structure to function. *Dev Biol (Basel)* 2005; 122: 117–27.
56. International Conference on Harmonisation. Specifications: Test procedures and acceptance criteria for biotechnological/ biological products. Q6B, March 1999.
57. Chirino AJ, Mire-Sluis A. Characterizing biological products and assessing comparability following manufacturing changes. *Nat Biotechnol* 2004; 22: 1383–91.
58. Drake AW, Myszkowski DG, Klakamp SL. Characterizing high-affinity antigen/antibody complexes by kinetic- and equilibrium-based methods. *Anal Biochem* 2004; 328: 35–43.
59. Cacia J, Keck R, Presta LG, et al. Isomerization of an aspartic acid residue in the complementarity-determining regions of a recombinant antibody to human IgE: identification and effect on binding affinity. *Biochemistry* 1996; 35: 1897–903.
60. Hunt G, Moorhouse KG, Chen AB. Capillary isoelectric focusing and sodium dodecyl sulfate-capillary gel electrophoresis of recombinant humanized monoclonal antibody HER2. *J Chromatogr* 1996; 744(1–2): 295–301.
61. Harris RJ, Kabakoff B, Macchi FD, et al. Identification of multiple sources of charge heterogeneity in a recombinant antibody. *J Chromatogr B Biomed Sci Appl* 2001; 752: 233–45.
62. Bai L, Burman S, Gledhill L. Development of ion exchange chromatography methods for monoclonal antibodies. *J Pharm Biomed Anal* 2000; 22: 605–11.

63. Santora LC, Krull IS, Grant K. Characterization of recombinant human monoclonal tissue necrosis factor- α antibody using cation-exchange HPLC and capillary isoelectric focusing. *Anal Biochem* 1999; 275: 98–108.
64. Perkins M, Theiler R, Lunte S, et al. Determination of the origin of charge heterogeneity in a murine monoclonal antibody. *Pharm Res* 2000; 17: 1110–17.
65. Weitzhandler M, Farnan D, Rohrer JS, et al. Protein variant separations using cation exchange chromatography on grafted, polymeric stationary phases. *Proteomics* 2001; 1: 179–85.
66. Dai HJ, Krull IS. Thermal stability studies of immunoglobulins using capillary isoelectric focusing and capillary zone electrophoresis methods. *J Chromatogr* 1998; 807: 121–8.
67. Kroon DJ, Baldwin-Ferro A, Lalan P. Identification of sites of degradation in a therapeutic monoclonal antibody by peptide mapping. *Pharm Res* 1992; 9: 1386–93.
68. Dillon TM, Bondarenko PV, Rehder DS, et al. Optimization of a reversed-phase high-performance liquid chromatography/mass spectrometry method for characterizing recombinant antibody heterogeneity and stability. *J Chromatogr* 2006; 1120: 112–20.
69. Chelius D, Rehder DS, Bondarenko PV. Identification and characterization of deamidation sites in the conserved regions of human immunoglobulin gamma antibodies. *Anal Chem* 2005; 77: 6004–11.
70. Chelius D, Jing K, Lueras A, et al. Formation of pyroglutamic acid from N-terminal glutamic acid in immunoglobulin gamma antibodies. *Anal Chem* 2006; 78: 2370–6.
71. Kotani N, Takasaki S. Analysis of 2-aminobenzamide-labeled oligosaccharides by high-pH anion-exchange chromatography with fluorometric detection. *Anal Biochem* 1998; 264: 66–73.
72. Clarke A, Harmon B, DeFelippis MR. Analysis of 3-(acetylamino)-6-aminoacridine-derivatized oligosaccharides from recombinant monoclonal antibodies by liquid chromatography–mass spectrometry. *Anal Biochem* 2009; 390: 209–11.
73. Ma S, Nashabeh W. Carbohydrate analysis of a chimeric recombinant monoclonal antibody by capillary electrophoresis with laser-induced fluorescence detection. *Anal Chem* 1999; 71: 5185–92.
74. Schon A, Velazquez-Campoy A. Calorimetry. In: Jiskoot W, Crommelin, DJA, eds. *Methods for Structural Analysis of Protein Pharmaceuticals*. Arlington: AAPS Press, 2005: 379–412.
75. Bloemendal M, Jiskoot W. Circular Dichroism Spectroscopy. In: Jiskoot W, Crommelin, DJA, eds. *Methods for Structural Analysis of Protein Pharmaceuticals*. Arlington: AAPS Press, 2005: 83–130.
76. Bloemendal M, Curtis Johnson Jr. W. Structural information on proteins from circular dichroism spectroscopy: possibilities and limitations. In: Herron JN, Jiskoot W, Crommelin, DJA, eds. *Physical Methods to Characterize Pharmaceutical Proteins*. New York: Plenum Press, 1995: 65–100.
77. Jiskoot W, Visser AJWG, Herron JN, et al. Fluorescence spectroscopy. In: Jiskoot W, Crommelin DJA, eds. *Methods for Structural Analysis of Protein Pharmaceuticals*. Arlington: AAPS Press, 2005: 27–82.
78. Jiskoot W, Hlady V, Naleway JJ, et al. Application of fluorescence spectroscopy for determining the structure and function of proteins. In: Herron JN, Jiskoot W, Crommelin DJA, eds. *Physical Methods to Characterize Pharmaceutical Proteins*. New York: Plenum Press, 1995: 1–63.
79. Weert M van de, Hering JA, Haris PI. Fourier transform infrared spectroscopy. In: Jiskoot W, Crommelin DJA, eds. *Methods for Structural Analysis of Protein Pharmaceuticals*. Arlington: AAPS Press, 2005: 131–66.
80. Cooper EA, Knutson K. Fourier transform infrared spectroscopy investigations of protein structure. In: Herron JN, Jiskoot W, Crommelin DJA, eds. *Physical Methods to Characterize Pharmaceutical Proteins*. New York: Plenum Press, 1995: 101–43.
81. Wishart W. Nuclear magnetic resonance spectroscopy. In: Jiskoot W, Crommelin DJA, eds. *Methods for Structural Analysis of Protein Pharmaceuticals*. Arlington: AAPS Press, 2005: 199–244.
82. Vander Velde DG, Matsuura J, Manning MC. Two-, three-, and four dimensional magnetic resonance spectroscopy of protein pharmaceuticals. In: Herron JN, Jiskoot W, Crommelin DJA, eds. *Physical Methods to Characterize Pharmaceutical Proteins*. New York: Plenum Press, 1995: 179–218.
83. Philo JS. Is any measurement method optimal for all aggregate sizes and types? *AAPS J* 2006; 8: E564–71.
84. Liu J, Andya JD, Shire SJ. A critical review of analytical ultracentrifugation and field flow fractionation methods for measuring protein aggregation. *AAPS J* 2006; 8: E580–9.
85. Philo JS. Analytical ultracentrifugation. In: Jiskoot W, Crommelin DJA, eds. *Methods for Structural Analysis of Protein Pharmaceuticals*. Arlington: AAPS Press, 2005: 379–412.
86. Mahler HC, Müller R, Friess W, et al. Induction and analysis of aggregates in a liquid IgG1-antibody formulation. *Eur J Pharm Biopharm* 2005; 59: 407–17.

87. Kiese S, Pappenberger A, Friess W, et al. Shaken, not stirred: mechanical stress testing of an IgG1 antibody. *J Pharm Sci* 2008; 97: 4347–66.
88. Demeester J., De Smedt SS, Sanders NN, et al. Light scattering. In: Jiskoot W, Crommelin DJA, eds. *Methods for Structural Analysis of Protein Pharmaceuticals*. Arlington: AAPS Press, 2005: 245–76.
89. International Conference on Harmonisation. Stability testing of new drug substances and products. Q1A(R2): February 2003.
90. International Conference on Harmonisation. Stability testing of biotechnological products. Q5C, November 1995.
91. International Conference on Harmonisation. Photostability testing of new drug substances and products. Q1B, November 1996.
92. International Conference on Harmonisation. Pharmaceutical development. Q8, May 2006.
93. International Conference on Harmonisation. Quality risk management. Q9, June 2006.
94. International Conference on Harmonisation. Comparability of biotechnological/biological products subject to changes in their manufacturing process. Q5E, June 2005.
95. Huang L, Lu J, Wroblewski VJ, et al. In vivo deamidation characterization of monoclonal antibody by LC/MS/MS. *Anal. Chem.* 2005; 77: 1432–9.
96. Lam XM, Oeswein JQ, Ongpipattanakul B, et al. Stabilized Antibody Formulation. Genentech, Inc., US Patent (2000) US 6991790 B1.
97. Jiskoot W, Beuvery EC, de Koning AAM, et al. Analytical approaches to the study of monoclonal antibody stability. *Pharm Res* 1990; 7: 1234–41.
98. Zheng JY, Janis LJ. Influence of pH, buffer species, and storage temperature on physicochemical stability of a humanized monoclonal antibody LA298. *Intl. J. Pharm.* 2006; 308: 46–51.
99. Wakankar AA, Borchardt RT, Eigenbrot C, et al. Aspartate isomerization in the complementarity-determining regions of two closely related monoclonal antibodies. *Biochemistry* 2007; 46: 1534–44.
100. Paborji M, Pochopin NL, Coppola WP, et al. Chemical and physical stability of chimeric L6, a mouse-human monoclonal antibody. *Pharm Res.* 1994; 11: 764–71.
101. Wei Z, Feng J, Lin HY, et al. Identification of a single tryptophan residue as critical for binding activity in a humanized monoclonal antibody against respiratory syncytial virus. *Anal Chem* 2007; 79: 2797–805.
102. Bertolotti-Ciarlet A, Wang W, Lownes R, et al. Impact of methionine oxidation on the binding of human IgG1 to Fc γ 1 and Fc γ 2 receptors. *Mol Immunol* 2009; 46: 1878–82.
103. Gaza-Bulsec G, Faldu S, Hurkmans K, et al. Effect of methionine oxidation of a recombinant monoclonal antibody on the binding affinity to protein A and protein G. *J Chromatogr B* 2008; 870: 55–62.
104. Liu D, Ren D, Huang H, et al. Structure and stability changes of human IgG1 Fc as a consequence of methionine oxidation. *Biochemistry* 2008; 47: 5088–100.
105. Taylor DM, Gibbs BF, Kabashi E, et al. Tryptophan 32 potentiates aggregation and cytotoxicity of a copper/zinc superoxide dismutase mutant associated with familial amyotrophic lateral sclerosis. *J Biol Chem* 2008; 282: 16329–35.
106. Sharp JS, Becker JM, Hettich RL. Analysis of protein solvent accessible surfaces by photochemical oxidation and mass spectrometry. *Anal Chem* 2004; 76: 672–83.
107. Usami A, Ohtsu A, Takahama S, et al. The effect of pH, hydrogen peroxide and temperature on the stability of human monoclonal antibody. *J Pharma Biom Anal* 1996; 14: 1133–40.
108. Zamani L, Andersson FO, Edebrink P, et al. Conformational studies of a monoclonal antibody, IgG1, by chemical oxidation: structural analysis by ultrahigh-pressure LC-electrospray ionization time-of-flight MS and multivariate data analysis. *Anal Biochem* 2008; 380: 155–163.
109. Shen FW; Kwong MY, Keck RG, et al. In: Marshak DR, ed. *Techniques in Protein Chemistry VII*. San Diego: Academic Press, 1996: 275–84.
110. Keck RG. The Use of t-butyl hydroperoxide as a probe for methionine oxidation in proteins. *Anal Biochem* 1996; 236: 56–62.
111. Chumsae C, Gaza-Bulsec G, Sun J, et al. Comparison of methionine oxidation in thermal stability and chemically stressed samples of a fully human monoclonal antibody. *J Chromatogr B* 2007; 850(1–2): 285–94.
112. Yang J, Wang S, Liu J, et al. Determination of tryptophan oxidation of monoclonal antibody by reversed phase high performance liquid chromatography. *J Chromatogr A* 2007; 1156(1–2): 174–82.
113. Lam XM, Yang JY, Cleland JL. Antioxidants for prevention of methionine oxidation in recombinant monoclonal antibody HER2. *J Pharm Sci* 1997; 86: 1250–5.

114. Hillgren A, Lindgren J, Aldén M. Protection mechanism of Tween 80 during freeze-thawing of a model protein, LDH. *Int J Pharm* 2002; 237: 57–69.
115. Bam NB, Cleland JL, Yang J, et al. Tween protects recombinant human growth hormone against agitation-induced damage via hydrophobic interactions. *J Pharm Sci* 1998; 87: 1554–9.
116. Jaspe J, Hagen SJ. Do protein molecules unfold in a simple shear flow? *Biophys J* 2006; 91: 3415–24.
117. Bee JS, Stevenson JL, Mehta B, et al. Response of a concentrated monoclonal antibody formulation to high shear. *Biotechnol Bioeng* 2009; 103: 936–43.
118. Colombié S, Gaunand A, Lindet B. Lysozyme inactivation under mechanical stirring: effect of physical and molecular interfaces. *Enzyme Microb Technol* 2001; 28: 820–6.
119. Henson AF, Mitchell JR, Musselwhite PR. The surface coagulation of proteins during shaking. *J Colloid Interface Sci* 1970; 32: 162–5.
120. Baszkin A, Boissonnade MM, Kamyshny A, et al. Native and hydrophobically modified human immunoglobulin G at the air/water interface. *J Colloid Interface Sci.* 2001; 239: 1–9.
121. Bee JS, Chiu D, Sawicki S, et al. Monoclonal antibody interactions with micro- and nanoparticles: adsorption, aggregation, and accelerated stress studies. *J Pharm Sci* 2009; 98: 3218–38.
122. Tyagi AK, Randolph TW, Dong A, et al. IgG particle formation during filling pump operation: a case study of heterogeneous nucleation on stainless steel nanoparticles. *J Pharm Sci* 2009; 98: 94–104.
123. Thirumangalathu R, Krishnan S, Ricci MS, et al. Silicone oil- and agitation-induced aggregation of a monoclonal antibody in aqueous solution. *J Pharm Sci* 2009; 98: 3167–81.
124. Shamlou PA, Breen LH, Bell WV, et al. A new scaleable freeze-thaw technology for bulk protein solutions. *Biotechnol Appl Biochem* 2007; 46 (Pt 1): 13–26.
125. Treuheit MJ, Kosky AA, Brems DN. Inverse relationship of protein concentration and aggregation. *Pharm Res* 2002; 19: 511–16.
126. Carpenter JF, Pikal MJ, Chang BS, et al. Rational design of stable lyophilized protein formulations: some practical advice. *Pharm Res* 1997; 14: 969–75.

15 | Stress testing of oligonucleotides

Daniel C. Capaldi

INTRODUCTION

The specific binding of synthetic oligonucleotides to cellular RNA through Watson–Crick base pairing can result in modulation of gene expression. This is the basis of the antisense concept, the therapeutic potential of which was first enunciated over 30 years ago by Zamecnik (1). The majority of oligonucleotides studied to date elicit their pharmacological effects by altering the metabolism of coding RNA (mRNA), that is, RNA that is ultimately translated into protein. Various steps in the processing of mRNA are amenable to intervention with antisense oligonucleotides, and oligonucleotides designed to alter splicing (2), to inhibit 5'-capping (3), and to prevent 3'-polyadenylation (4) have all demonstrated activity *in vivo*, but the most studied group of antisense oligonucleotides is that designed to promote RNase H-mediated cleavage of mRNA. In addition to mRNA targets, the recent realization that noncoding RNA plays a more important role regulating protein expression than was previously suspected has opened up new classes of RNA as potential therapeutic targets (5). Finally, while the majority of therapeutic oligonucleotides seek to manipulate gene expression (by targeting RNA), oligonucleotides designed to modulate the immune response (6) and oligonucleotides that bind to protein targets by virtue of possessing discrete three-dimensional structures [called aptamers (7)] are also the subjects of intense clinical research and development.

A variety of chemically modified oligonucleotides has been evaluated as potential therapeutics. Oligonucleotides possessing modified heterocyclic bases (8), sugars, and internucleotide linkages (9) and oligonucleotides conjugated to polymers such as polyethylene glycol, cholesterol, and fatty acids (10) have been explored in the search for molecules that are both potent and well tolerated. To date, most of the molecules used in the therapeutic setting have been single stranded; however, recent advances in RNA biology, which culminated in the award of the Nobel Prize in Physiology or Medicine to Fire and Mellow in 2006, have spurred clinical evaluation of a variety of double-stranded molecules that aim to exploit the RNA interference (RNAi) pathway (11).

Arguably the best studied class of therapeutic oligonucleotides is the 2'-deoxyphosphorothioate diester class (II, Fig. 1), whose use as therapeutic agents was pioneered in the 1990s by Isis Pharmaceuticals, Inc., and Genta (12).

2'-Deoxyphosphorothioate diester oligonucleotides differ from natural DNA (I) in that one of the nonbridging oxygen atoms of the internucleotide linkages of the latter is replaced by a sulfur atom. This simple modification imparts several pharmacologically useful properties, among which is improved stability toward enzymatic degradation. Additionally, the sulfur atom confers a pharmacokinetic benefit by significantly increasing binding to plasma proteins, preventing rapid renal excretion. The substitution of a nonbridging oxygen atom for a sulfur atom is also notable for preserving in 2'-deoxyphosphorothioate diester oligonucleotides an important property of DNA, i.e., the Watson–Crick duplexes formed between the antisense oligonucleotide and its target RNA molecule are substrates for RNase H. This property results in RNase H-mediated cleavage of target RNA, which is the best characterized and most rugged terminating mechanism identified to date (13) and critical to the success of most antisense drugs.

Although 2'-deoxyphosphorothioate oligonucleotides are more enzymatically stable than DNA, their tissue half-lives, which can normally be measured in hours (14), are still relatively short. Furthermore, because the substitution of oxygen for sulfur results in a decrease in affinity for RNA (15), 2'-deoxyphosphorothioate oligonucleotides are probably insufficiently potent to warrant development in many indications. To address these limitations, Isis developed second-generation phosphorothioate diester antisense oligonucleotides in which a number of the 2-deoxyribose sugars of the first-generation molecules are replaced by 2-O-(2-methoxyethyl)

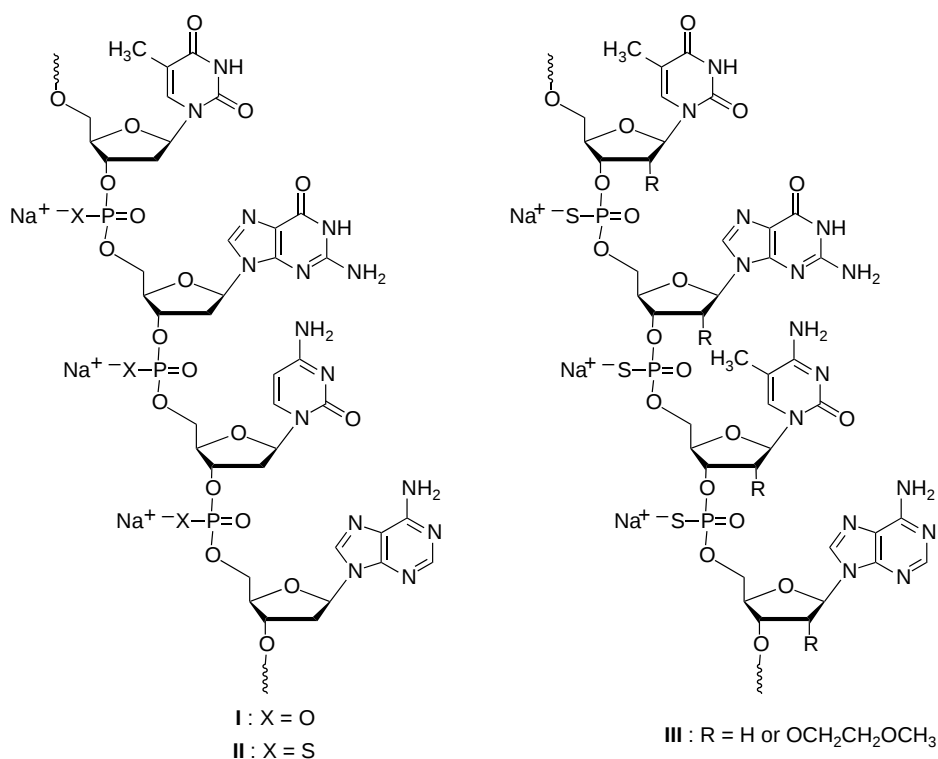


Figure 1 DNA (I); 2'-deoxyphosphorothioate diester oligonucleotide (II) and 2'-O-(2-methoxyethyl) modified phosphorothioate diester oligonucleotide (III).

ribose sugars (III, Fig. 1). The 2'-O-(2-methoxyethyl), or MOE modification, conveys considerable extra protection from nuclease digestion (16). Additionally, the presence of the 2-methoxyethoxy group in the 2-position forces the sugar into a northern, C3'-endo conformation, increasing the affinity of the antisense oligonucleotide for its target (15). Unfortunately, while oligonucleotides uniformly modified with 2'-O-(2-methoxyethyl)ribose sugars are exceptionally stable toward nuclease degradation, the high-affinity duplexes they form with RNA are not substrates for RNase H, which severely limits their usefulness as antisense constructs. However, much of the stability and affinity of uniformly modified MOE oligonucleotides is preserved within molecules consisting of short runs of MOE nucleotides located at the 5' and 3' ends that subtend a gap of 2'-deoxyphosphorothioate nucleotides (Fig. 2), which form duplexes with target RNA that are recognized by RNase H.

As a result of their improved stability, their increased affinity for target and by virtue of their ability to support RNase H when bound to RNA, 2'-MOE/2'-deoxyphosphorothioate diester oligonucleotides, which are often simply referred to as MOE gapmers, display antisense potencies between 10 and 50 times those of 2'-deoxyphosphorothioate diester oligonucleotides (17). Equally importantly, when compared to 2'-deoxyphosphorothioate diester oligonucleotides, MOE gapmers are at least fivefold less immunostimulatory in mice (18) and display decreased anticoagulant effects and show less potential for complement activation in nonhuman primates (19). The combination of improved potency and decreased toxicity suggests that MOE gapmers will become important weapons in the fight against a variety of disorders (20).

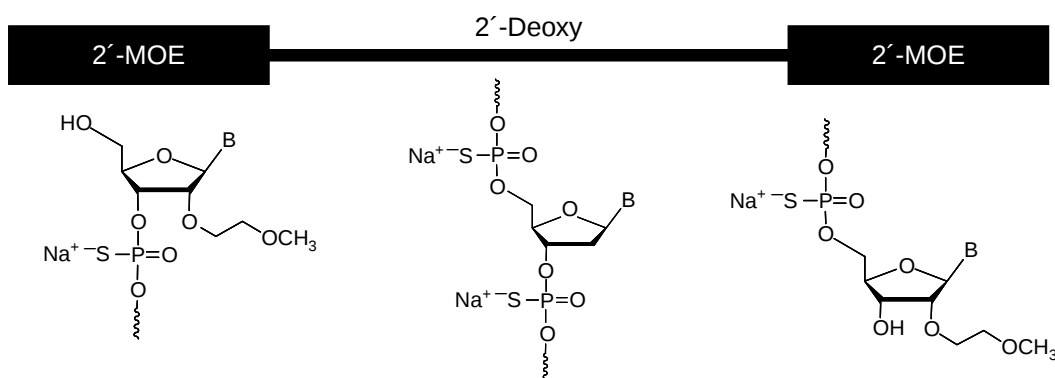


Figure 2 Cartoon showing arrangement of 2'-MOE and 2'-deoxynucleosides in gapmer antisense constructs (B = adenin-9-yl, guanin-9-yl, thymine-1-yl or 5-methylcytosin-1-yl).

None of the exciting clinical progress made with MOE gapmers could have occurred absent a detailed understanding of their pharmacological and toxicological properties. However, just as important to the current and continued clinical progress of these molecules, and ultimately to their approval and commercialization as therapeutics, were efforts devoted to improving manufacturing processes (21) and to understanding their physical and chemical properties. In regard to the latter, a particularly important undertaking was the determination of the inherent stability of MOE gapmers and the development of an understanding of their degradation pathways and degradation products. In this chapter, I describe the main degradation pathways, the structures of degradation products and the inherent stabilities of MOE gapmers exposed to a variety of physical and chemical insults. While the results presented were obtained using MOE gapmer phosphorothioate oligonucleotides, it is likely other oligonucleotides that contain identical or similar heterocyclic bases, sugars or internucleotide linkages will behave along the same lines.

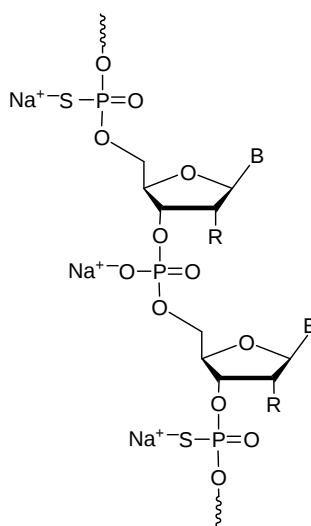
Before entering into a discussion of the results obtained under the various stress conditions, it is perhaps worth devoting some space to the main analytical technique that is used at Isis to detect and quantify degradation products.

ANALYTICAL METHODOLOGY

A variety of electrophoretic, chromatographic, and spectroscopic techniques for determining the stability of oligonucleotides has been described. At Isis, early separations of clinical materials were accomplished using denaturing polyacrylamide gel electrophoresis (PAGE) with quantitation by UV densitometry. In the late 1980s, capillary gel electrophoresis (CGE) methods capable of resolving nucleic acids of differing lengths were introduced. These methods were adapted for the analysis of phosphorothioate oligonucleotides by increasing the theoretical plate count of the gel-filled capillary (22,23).

In addition to length-based degradation products, which may be detected by CGE, phosphorothioate diester oligonucleotides normally contain low levels of full-length molecules that contain a phosphate diester linkage in place of a phosphorothioate diester linkage. Often termed (P=O)₁, this component (IV, Fig. 3), which is a process-related substance and a degradation product, can be resolved from the fully thioated parent molecule using strong anion exchange (SAX) chromatography (24,25).

Prior to 2000, we determined the stability of phosphorothioate oligonucleotides using a combination of CGE for separation of length-based degradation products and SAX HPLC for control of (P=O)₁. This meant, of course, that every sample had to be analyzed by two separate methods, which added cost, especially in the QC environment where each method had



IV

Figure 3 $(P=O)_1$ component of a MOE gapmer oligonucleotide. $(P=O)_1$ is related to the parent sequence by exchange of any one of its phosphorothioate diester linkages for a phosphate diester linkage. (B = adenin-9-yl, guanin-9-yl, thymine-1-yl or 5-methylcytosin-1-yl; R = H or $OCH_2CH_2OCH_3$).

standards and system suitability checks and each instrument qualification and maintenance requirements. A more serious drawback, however, was that the combination of SAX HPLC and CGE was insufficiently selective to detect and quantify many of the degradation products of phosphorothioate oligonucleotides.

HPLC-mass spectrometry (HPLC-MS) is a powerful tool for the analysis of single- and double-stranded oligonucleotides. Of the two commonly used ionization techniques suitable for oligonucleotide analysis, that is, matrix-assisted laser desorption-ionization (MALDI) and electrospray ionization (ESI), the ability of the latter to produce ions directly from a flowing solution renders it more suited to chromatographic hyphenation. Both reversed phase (RP) and ion-pair (IP) mobile phases are compatible with ESI-MS, although the increased resolving power of IP chromatography makes it the preferred separation mode for HPLC-MS of oligonucleotides (26). In recent years, IP-HPLC-ESI-MS has been used, among other things, for analysis of phosphorothioate oligonucleotides (27,28) and their metabolites (29–32) and for analysis of the ocular metabolites of siRNA duplexes (33). The lack of specificity inherent in the combination of CGE and SAX HPLC prompted us to examine IP-HPLC-ESI-MS as a method for the routine stability analysis of phosphorothioate oligonucleotides.

There are several important issues associated with any attempt to use IP-HPLC-ESI-MS for the quantitative analysis of oligonucleotides. The first of these is that under typical HPLC-ESI-MS conditions, the MS signal due to the oligonucleotide is distributed across several charge states. This complicates the analysis and reduces the signal strength in any one charge state. Charge state distribution in ESI-MS is influenced by factors such as solution pH and the concentration and identity of the ion-pairing reagent (26). With this in mind, several trialkyl ammonium buffers were evaluated for their influence on the charge state distribution of a 20-mer phosphorothioate oligonucleotide. The results from these studies indicated that by using 5 mM tributylammonium acetate (TBAAC) in water–acetonitrile mixtures, we could force between 70% and 80% of the parent compound into the -4 charge state (34), thus simplifying the analysis and improving sensitivity.

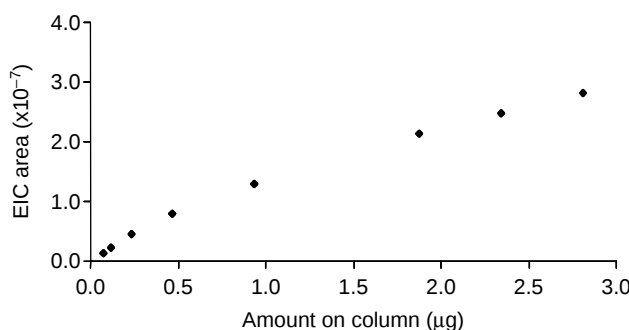


Figure 4 Relationship between MS response and column load for a MOE gapmer.

In addition to its simplifying effect on charge state distribution, TBuAA is a more effective IP reagent than, for example, triethylammonium acetate (TEAA), which means it provides for better retention of the oligonucleotide at the same concentration. The benefits of this are twofold. First, good separations can be achieved using low concentrations of IP reagent and second, high concentrations of acetonitrile are required to elute the sample from the column. The low IP reagent concentration and high organic content of the electrospray feed combine to increase ionization efficiency.

A second issue associated with using mass spectrometry for quantitative analysis of oligonucleotides is that molecules of different lengths ionize with different efficiencies, that is, short oligonucleotides ionize more readily than long oligonucleotides. Unless the relative ionization efficiencies are known a priori, one risks overestimating the amount of shorter and underestimating the amount of longer components present in the sample. To avoid establishing response factors for components of different lengths, we elected to restrict the use of MS quantification to those components that are not resolved chromatographically from the parent compound and use UV detection to quantify the remainder. Under our IP-HPLC conditions, the main UV peak of 20-mer MOE gapmers (Fig. 5) contains oligonucleotides that are between one nucleotide shorter and one nucleotide longer than the parent compound. We have demonstrated through recovery experiments (vide infra) that the ionization efficiencies of these, the smallest and largest components that are quantified by MS, are essentially the same as that of the parent oligonucleotide.

A third challenge inherent to using mass spectrometry in a quantitative sense is that unlike UV response, which is linear at analytically relevant concentrations, the MS response plateaus as the analyte concentration increases. This is because, as concentration increases, there is insufficient space on a single electrospray droplet to accommodate in a linear fashion more and more molecules for ionization (35). To illustrate the effect, the response due to the full length, fully thioated parent component of a MOE gapmer is plotted against column load in Figure 4.

Why is the effect important? First, degradation products are present at much lower levels than the parent oligonucleotide. Second, with the exception of the $(P=O)_1$ component, degradation products are partially resolved chromatographically from the parent component. This means that the amount of analyte entering the mass spectrometer is lower for the degradation products than for the parent and $(P=O)_1$ components, which in turn means the responses due to the degradation products fall into the linear section of the response curve while those due to the parent and $(P=O)_1$ component do not. Unless this is taken into account, any determinations of low-level components that are based on a comparison of ion current areas will be overestimates.

Of course, the nonlinearity of a response does not *de facto* preclude its use for determining analyte levels. Instead, what is required is that the relationship between analyte concentration

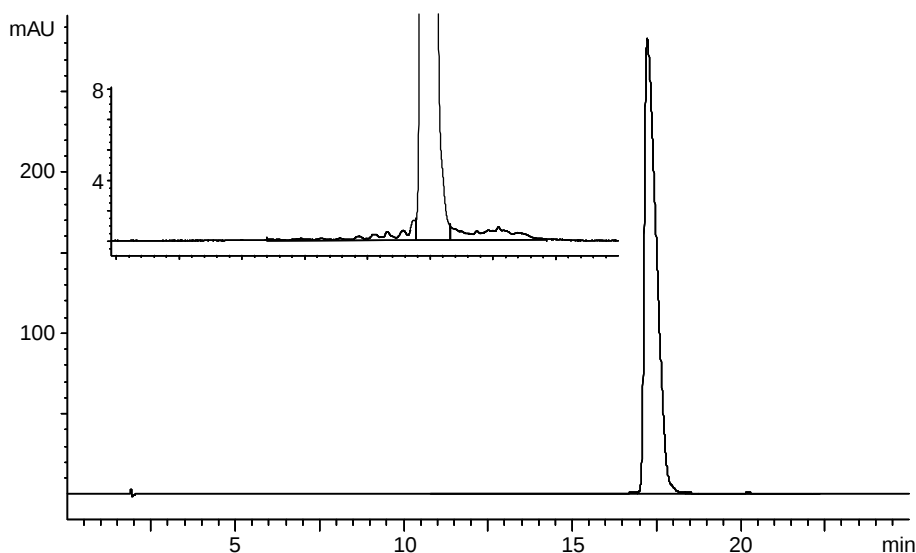


Figure 5 Full scale and expanded view of IP-HPLC-UV chromatogram of a 20-mer MOE gapmer.

and response be understood. In the present case, we realized that the change in MS response with increasing oligonucleotide concentration could be described by a quadratic equation, which could then be used to interpret the MS responses due to each component of the sample accurately. The procedure is as follows: a four-point calibration curve is generated by injecting various volumes of a standard solution of oligonucleotide. The MS responses due to the parent and $(P=O)_1$ components are integrated. The summed responses, parent plus $(P=O)_1$, for each injection are plotted against injection volume and the resulting points fit by a second-order polynomial equation, which is used to interpret the MS responses due to the other components of the sample. These values, which are in effect linearized MS responses, are compared to each other to determine the relative amounts of each component present.

As a result of the method development efforts described above, we were able to replace our CGE-SAX HPLC combination with IP-HPLC-ESI-MS for stability (and release) analysis of all phosphorothioate oligonucleotide drug substances and drug products (36). Assay and purity analysis of a batch of MOE gapmer drug substance follows the route described below.

An aqueous solution of drug substance is chromatographed on a C18 column held at 50°C and eluted with a gradient of acetonitrile in aqueous 5 mM TBuAA at a flow rate of 0.25 mL/min. A typical UV chromatogram is shown in Figure 5.

Inspection of the UV chromatogram (expanded view) reveals some earlier and some later eluting components, which can be quantified using UV detection. After passing through the UV detector, the eluate is fed directly into a single quadrupole ESI mass spectrometer. The mass window is set to extend from 150 amu below to 150 amu above the calculated most abundant mass of the -4 charge state of the parent oligonucleotide, which is sufficient to permit detection of all components that elute within the main UV peak. The average mass spectrum of the main UV peak of the material shown in Figure 5 is shown in Figure 6.

In this example, the full-scale mass spectrum (shown inset in Fig. 6) shows a peak at $m/z = 1793$ with relative abundance = 100%, which is consistent with the value calculated for the -4 charge state of the most abundant mass of the parent oligonucleotide. When the y -axis is expanded, it is evident that the main UV peak shown in Figure 5 contains other components that, although not sufficiently chromatographically resolved from the parent oligonucleotide to be evident in the UV chromatogram, are detected in the average mass spectrum because they

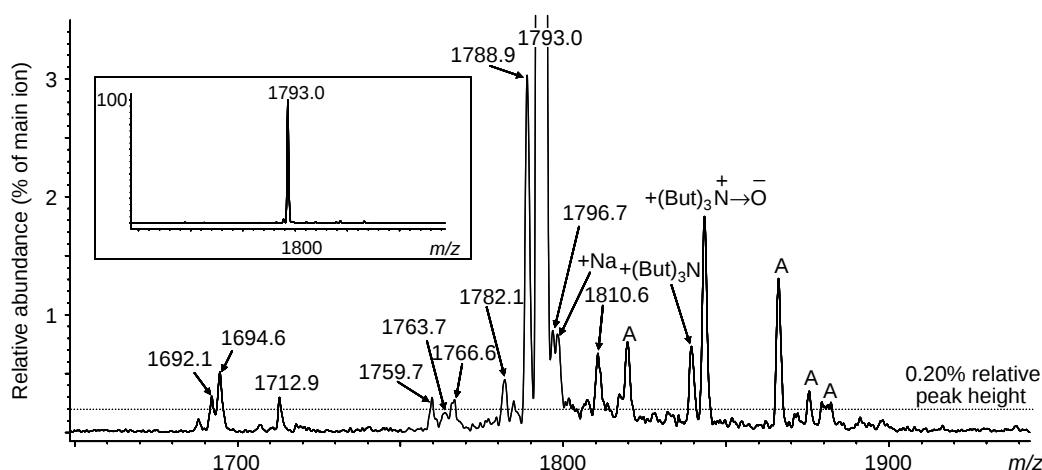


Figure 6 Expanded average mass spectrum of main UV peak of Figure 5 with full-scale spectrum shown inset.

have different most abundant masses (m/z values for these components are shown in Figure 6). The ability to detect components that are not chromatographically well resolved from the parent by simultaneously monitoring the MS dimension is what gives the method its enhanced selectivity. Clearly, in order to be useful for this purpose, the average mass spectrum must exhibit a high signal-to-noise ratio. Inspection of Figure 6 reveals that under the conditions described, components present at 0.20% of the parent component are easily distinguishable from background. It may also be noted that the average mass spectrum displays only low levels of the noncovalent sodium and buffer adducts (identified by name or with the letter A in Figure 6) that often plague MS analysis of oligonucleotides. For example, the sodium adduct in this spectrum has a relative abundance of only ca. 0.8%.

In the next step of the analysis, the ion currents due to each of the components present under the main UV peak are extracted; ion currents due to metal and buffer adducts of the parent component are not extracted. The extraction process results in a series of ion chromatograms, one for each ion extracted, which are then integrated. Representative ion chromatograms are shown in Figure 7.

As noted, because the MS response is not linear, direct comparison of ion current areas leads one to overestimate levels of components that are present at low concentrations. To avoid this, the responses are linearized using a quadratic curve produced by plotting ion current against injection volume for a reference standard. Once linearized, the ion current areas of the sample are compared one to another to determine the relative amounts of each component contained within the main UV peak. To determine the contribution of each component to the total sample, the results are simply multiplied by the proportion of the total UV absorbance accounted for by the main UV peak.

The accuracy, precision, and robustness and the limits of detection (LOD) and quantitation (LOQ) of this approach have been determined (36). Accuracy was evaluated by analyzing samples of oligonucleotide drug substance to which were added known quantities of various unambiguously synthesized degradation products and process-related substances. Example recovery plots, that is, plots of amount measured against amount added, for two degradation products, $(P=O)_1$ (Fig. 3) and a 3-(2-oxopropyl)imidazo(1,2-c)pyrimidin-5-methyl-6H-one (OIP)-modified oligonucleotide [Fig. 19 (37)], are shown in Figure 8. The results shown in Figure 8 are typical of those obtained for all components tested to date. We find that R^2 values are high, slopes are close to 1 and y -intercepts are either negligible, or, as is the case of species such as $(P=O)_1$ that are present as process-related substances, good estimates of native levels.

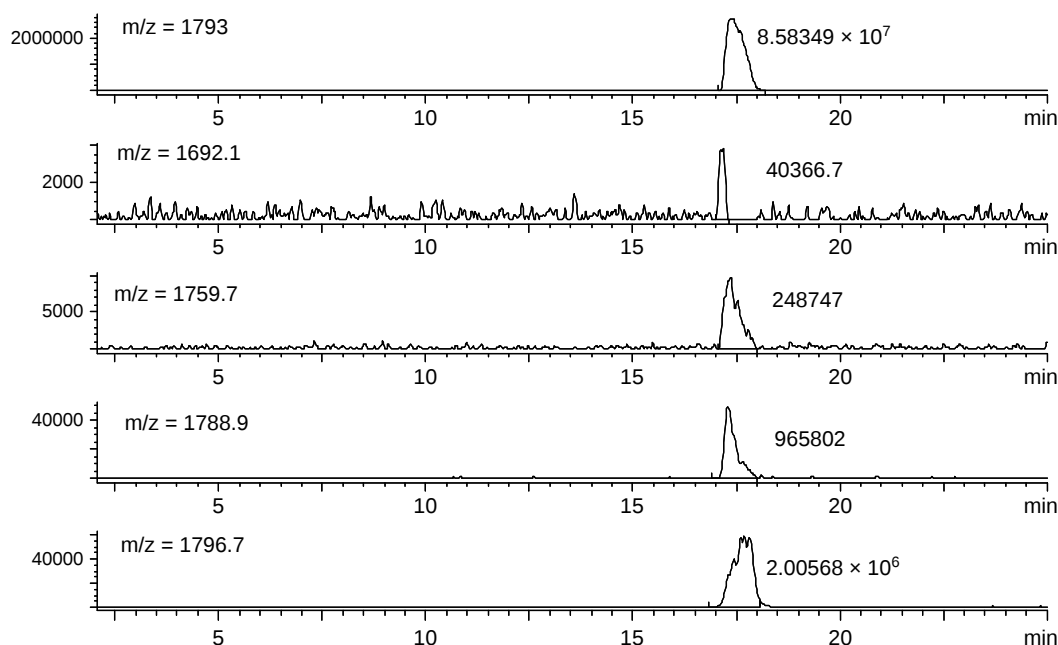


Figure 7 Extracted ion chromatograms for $m/z = 1793.0, 1692.1, 1759.7, 1788.9,$ and 1796.7 .

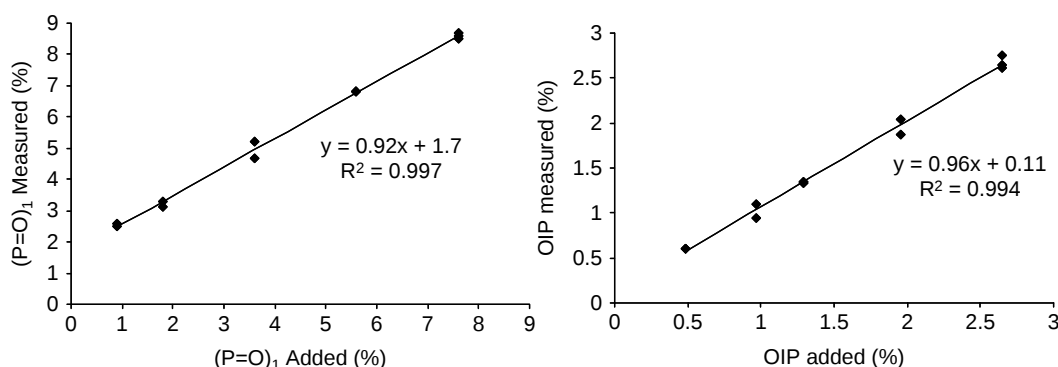


Figure 8 Recovery plots for $(P=O)_1$ (Fig. 3) and 3-(2-oxopropyl)imidazo(1,2-c)pyrimidin-5-methyl-6H-one (OIP, Fig. 19) degradation products.

These data suggest that the IP-HPLC-ESI-MS method developed at Isis is suitable for detecting and quantifying low levels of process-related substances and degradation products that are not easily detected and quantified by more traditional electrophoretic and chromatographic methods used to analyze oligonucleotides.

Over the last 8 years, we have used IP-HPLC-ESI-MS to detect and quantify a variety of process-related substances and degradation products of phosphorothioate oligonucleotides. It is true that several of these components have been described previously and are quantifiable, at least in some approximate fashion, by more traditional analytical techniques; the use of SAX chromatography to detect and quantify the $(P=O)_1$ component is one example. In other cases, for example, depurinated oligonucleotides (section "Acid Stress"), implementation of IP-HPLC-ESI-MS permitted for the first time quantification of components that while

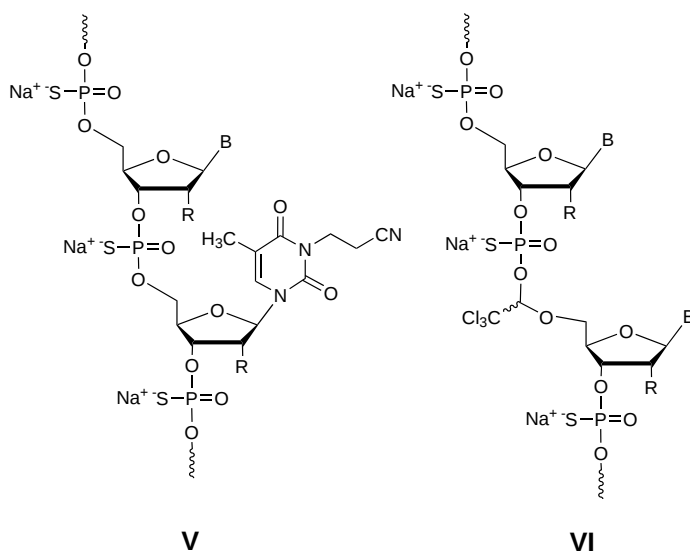


Figure 9 CNET (**V**) and trichloroacetaldehyde (**VI**) process-related substances of MOE gapmer oligonucleotides. (B = adenin-9-yl, guanin-9-yl, thymin-1-yl or 5-methylcytosin-1-yl; R = H or $\text{OCH}_2\text{CH}_2\text{OCH}_3$).

anticipated, had hitherto escaped detection. The real power of the method, however, lies in its ability to detect, quantify, and even provide some rudimentary structural information (the act of detection provides a low-resolution molecular mass) for previously unknown components. In some cases, this new knowledge spurred manufacturing changes that improved drug substance purity. For example, IP-HPLC-ESI-MS analysis of older lots of drug substance revealed that they contained substantial amounts (up to 5%) of a component with a molecular weight of 53 amu more than the parent compound, which we later discovered contained an N^3 -(2-cyanoethyl)thymine (CNET, **V**, Fig. 9) residue in place of a thymine residue. We demonstrated that this component, which forms when 2-cyanoethyl-protected oligonucleotides are treated with ammonium hydroxide, could be completely avoided by pretreatment with triethylamine (38).

In another example, analysis of some lots of drug substance showed the presence of a related substance with a most abundant mass 147 amu greater than the parent, which we demonstrated contained the atoms of trichloroacetaldehyde inserted into an internucleotide linkage [**VI**, Fig. 9 (39)]. This component forms due to the presence of low levels of trichloroacetaldehyde in some lots of dichloroacetic acid (DCA). In both the CNET and trichloroacetaldehyde examples, IP-HPLC-ESI-MS not only enabled discovery of the components, but also guided process development efforts aimed at their avoidance.

Finally, and most relevantly in the context of this chapter, IP-HPLC-ESI-MS has proven indispensable in our efforts aimed at establishing degradation pathways, degradation product structures, and the inherent stability of phosphorothioate gapmer oligonucleotides exposed to a range of stress conditions. In the sections below, we examine the impact of acidic, oxidative, basic, thermal, and photolytic stress conditions on MOE gapmer phosphorothioate oligonucleotides.

ACID STRESS

The results of exposure to conditions of low pH were reported among the course of the first investigations ever performed on nucleic acids (40). Exposure to acid results in cleavage of the nucleoside glycosidic bond and release of the heterocycle. The susceptibility of the glycosidic bond toward acid hydrolysis is dependent on the structure of both the sugar and the aglycone. 2'-Deoxyribonucleosides are hydrolyzed between 100 and 1000 times more quickly than the corresponding ribonucleosides (41–43), and purine nucleosides are hydrolyzed at

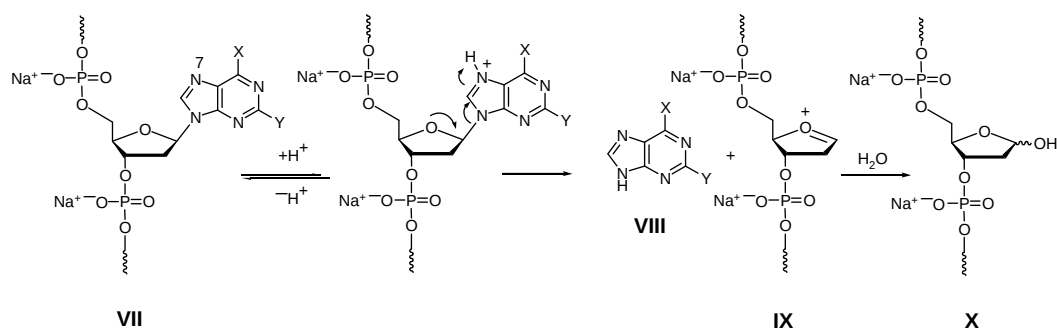


Figure 10 Depurination of DNA (X = OH, Y = NH₂ or X = NH₂, Y = H).

least 20 times faster than pyrimidines. As a consequence, the glycosidic bond is significantly more acid sensitive in DNA than it is in RNA and when exposed to acid, DNA depurinates much more rapidly than it depyrimidinates. RNA is also sensitive to acid, but it suffers internucleotide cleavage and phosphoryl migration significantly more quickly than glycolysis (44). The products of depurination of DNA (VII) are a nucleobase VIII (guanine or adenine) and a molecule that contains an apurinic site X [Fig. 10 (45)]. Depurination rates have been measured under a variety of conditions. The reaction is acid catalyzed and is thought to involve initial protonation of N⁷, which greatly increases the leaving group ability of the purine base. The initially formed oxonium ion IX reacts with water to give an oligonucleotide containing a 2-deoxyribose residue X.

In light of what is known about DNA, it was perhaps not surprising to discover that MOE gapmer oligonucleotides are susceptible to degradation under acidic conditions. The results obtained by IP-HPLC-ESI-MS analysis of a typical experiment, in which a pH 3.0 solution of 20-mer MOE gapmer of sequence ^{Me}U^{Me}C^{Me}C^{Me}CATT^{Me}CAGGAG^{Me}C^{Me}C^{Me}UGG (1)¹ was incubated at 25°C for 7 hours, are shown in Figure 11 (46).

Inspection of the UV chromatogram of the acid-treated sample [Fig. 11(B)] showed the presence of two early eluting peaks (*t_R* = ca. 2 minutes) that were absent from the control. These peaks likely represent adenine and guanine, which were hydrolyzed from the oligonucleotide upon exposure to acid. The remainder of the UV chromatogram of the acid-treated sample was very similar to that of the control, which suggested that treatment with acid does not result in cleavage of internucleotide linkages and formation of shorter oligonucleotides.

The UV chromatogram, however, provides only a partial picture of the degradation suffered by the parent oligonucleotide under these conditions. A much fuller description is available by considering not only the UV portion of the analysis, but also the MS dimension (shown in the inset in Fig. 11). Regarding the latter, it is clear that the average mass spectrum of the main UV peak of the acid-treated sample [inset in Fig. 11(B)] contains much higher levels of components with *m/z* = 1701.3 and 1697.2 than the control. These signals are consistent with loss of adenine from the parent molecule followed by the addition of water (calc. *m/z* = 1701.2) and loss of guanine followed by addition of water (calc. *m/z* = 1697.2), a result that confirmed the parent sequence suffered depurination upon treatment with acid to give components that contain an abasic site (analogous to X in Fig. 10). In this example, the extent of depurination in the acid-treated sample was such that parent oligonucleotide that had undergone two depurination events was also observed (*m/z* = 1671.8, 1668.1 and 1664.0 are consistent with molecules that have each gained two molecules of water and lost either two adenines (calc. *m/z* = 1672.0),

¹Underlined letters are 2'-MOE residues; all internucleotide linkages are phosphorothioate diesters.

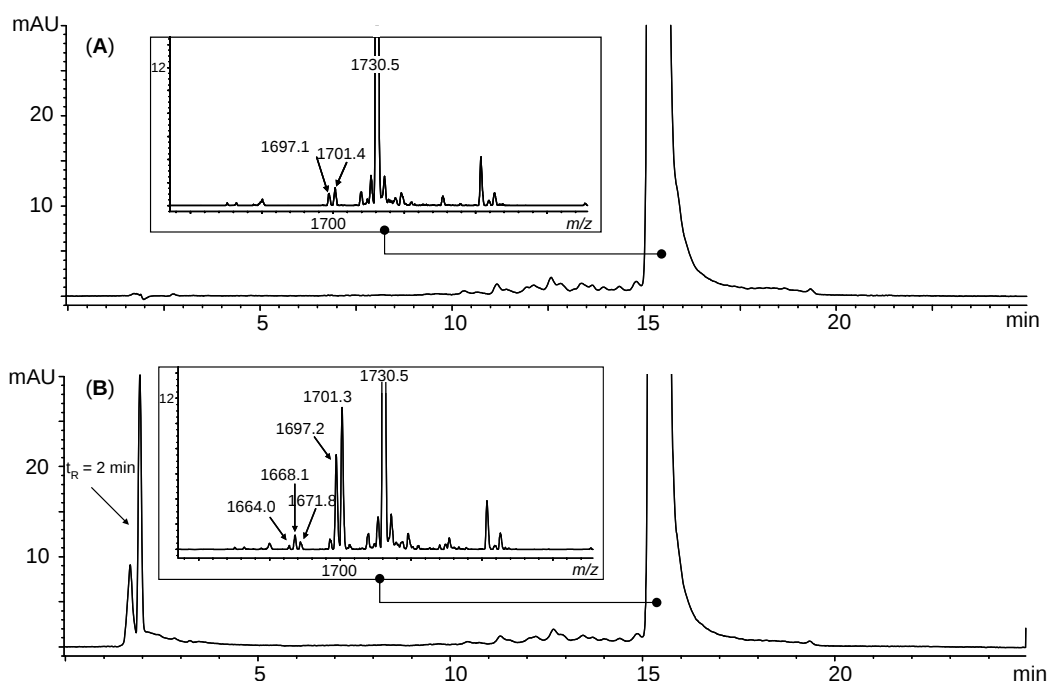


Figure 11 IP-HPLC-UV and (inset) ESI-MS results of (A) control and (B) acid stressed MOE gapmer **1** (pH 3.0, 25°C, 7 hours).

Table 1 Acid Stress of MOE Gapmer **1**

<i>m/z</i> (Observed)	Description	Percent of Sample	
		Control	Acid Treated
1730.5	Parent oligonucleotide (1)	90.2	78.4
1701.3	Parent – adenine + water	0.88	7.1
1697.2	Parent – guanine + water	0.71	4.7
1671.8	Parent – 2 × adenine + 2 × water	ND ^a	0.36
1668.1	Parent – adenine – guanine + 2 × water	ND	0.66
1664.0	Parent – 2 × guanine + 2 × water	ND	0.15

^aNone detected (lower limit of detection = 0.1%).

an adenine and a guanine (calc. m/z = 1668.0) or two guanines (calc. m/z = 1664.0), respectively). The relative amounts of depurinated and parent oligonucleotide were quantified as described in section “Analytical Methodology”, by integration of the extracted ions chromatograms due to each component present in the sample. The results of the analysis are provided in Table 1.

The data presented in Figure 11 and Table 1 clearly show one advantage that IP-HPLC-ESI-MS enjoys over HPLC-UV for the analysis of oligonucleotides. That is, while one can detect liberation of purine bases by inspection of the UV chromatogram alone, the enhanced selectivity afforded by MS permits detection and quantification of components that do not resolve chromatographically from the parent; it is only by looking at both the UV and MS dimensions that the true extent of degradation, ca. 15% in this case, is evident.

As expected, the sensitivity of MOE gapmers to acid increases with increasing 2'-deoxy-purine content. Also in line with expectations, under these conditions (ca. pH 3 and 25°C), proclivity toward depurination does not correlate with 2'-MOE purine content. This is consistent with the hypothesis that ribonucleoside-derived MOE purine residues are considerably more resistant to acid treatment than 2'-deoxypurine residues, which suggests that depurination of MOE gapmers exposed to mildly acidic conditions is confined to the 2'-deoxypurine residues of the gap.

OXIDATIVE STRESS

The heterocyclic bases of DNA and RNA are susceptible to oxidative damage. A panoply of oxidation products has been described (see Fig. 12 for some examples), the in-depth discussion of which is beyond the scope of this chapter (47–49). Research in this area is driven by the fact that reactive oxygen species (ROS), e.g., the superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($HO\cdot$), hydrogen peroxide, and reactive nitrogen species, e.g., peroxynitrite (ONO_2^-) are produced endogenously by oxidative cellular metabolism, and these and other oxidants probably represent the most common insult to which cellular DNA is exposed. It is believed that oxidative degradation of DNA is involved in aging processes and in several human diseases (50–52).

The sugar residues of DNA and RNA are also susceptible to oxidative damage (53). Studies have demonstrated that all of the hydrogen atoms of 2-deoxyribose and ribose are potential targets and that hydrogen atom abstraction frequently produces oxidized abasic sites as well as strand breaks.

Anticipating MOE gapmers may be rather sensitive toward oxidative stress, we elected to expose a sample to dilute H_2O_2 , which, in the absence of metals or other factors that catalyze formation of hydroxyl radicals through Fenton-type processes, is quite unreactive toward DNA (54). To this end, a sample of **1** (section "Acid Stress") was dissolved in 0.03% aqueous H_2O_2 solution. The resulting solution was held for 2 h at 25°C and then analyzed by IP-HPLC-ESI-MS. The results of the analysis are shown in Figure 13 (55).

Unlike phosphate diester DNA, it is clear from the data presented in Figure 13 that phosphorothioate MOE gapmers such as **1** are rather sensitive toward H_2O_2 . Degradation appears to result in formation of two types of product. Within the main UV peak [Fig. 13(A), t_R = 15–16.5 min], we noted an increase in levels of a component with m/z = 1726.6 and the appearance of another component with m/z = 1722.3 that was absent from the control sample [cf. Fig. 13(B) and 13(C)]. These components have most abundant masses that are 15.6 and 32.8 Da smaller than **1**, respectively. The signal at m/z = 1726.6 is almost certainly due to a group of components that are related to the parent compound by substitution of one of the sulfur atoms of the phosphorothioate backbone for oxygen. In the case of **1**, each of these components, which are often collectively referred to as $(P=O)_1$ (Fig. 3), contain 18 phosphorothioate diester linkages and one phosphate diester linkage. As it is probable that any one of 19 internucleotide linkages may be effected, the $(P=O)_1$ component of **1** is likely 19 different compounds. The signal at m/z = 1722.3 is probably due to a group of components that contain two phosphate diester linkages and 17 phosphorothioate linkages. This group, which is sometimes referred to collectively as

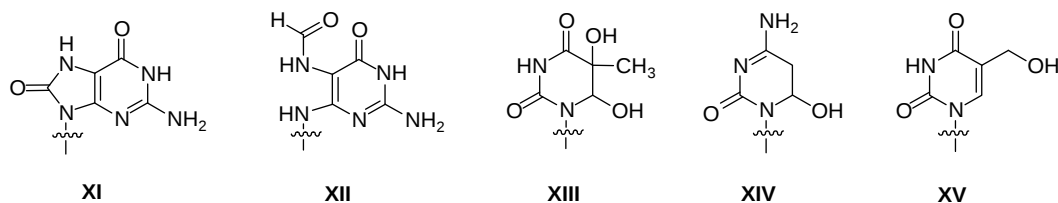


Figure 12 Some of the more important modified bases produced by exposure of DNA to reactive oxygen and nitrogen species: 8-oxoguanine (**XI**); 2,6-diamino-4-hydroxy-5-formamidopyrimidine (**XII**); thymine glycol (**XIII**); 6-hydroxy-5,6-dihydrocytosine (**XIV**); 5-hydroxymethyluracil (**XV**).

(P=O)₂, presumably forms by hydrolysis of the (P=O)₁ component and is likely a collection of 342 (i.e., 19 × 18) different species. The relative levels of **1** and its (P=O)₁ and (P=O)₂ degradates were estimated by extracting and integrating the corresponding ion current signals. The results of the analysis are provided in Table 2.

As alluded to above, the observed desulfurization of MOE gapmers upon exposure to H₂O₂ was not unexpected and was consistent with literature reports describing desulfurization of phosphorothioate triesters under similar conditions (56). The desulfurization reaction presumably proceeds by initial electrophilic attack of H₂O₂ on the sulfur atom of an internucleotide linkage to form **XVI**, which may then collapse to pentacoordinate intermediate **XVII** before losing sulfur to give the corresponding phosphate diester **XVIII** (path a, Fig. 14).

In addition to differences in levels of phosphate diesters, which co-elute with **1**, inspection of the IP-HPLC-UV chromatograms [Fig. 13(A)] revealed an increase in earlier eluting components following exposure to hydrogen peroxide. The increase indicated H₂O₂ treatment

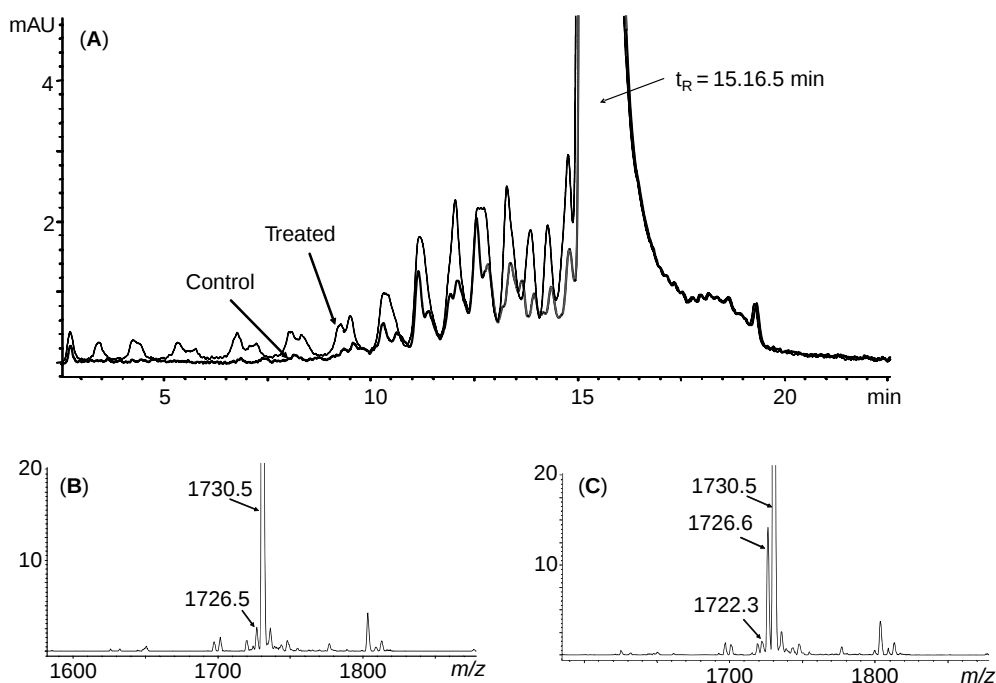


Figure 13 Oxidative stress of MOE gapmer **1**: (A) IP-HPLC-UV chromatogram; (B) ESI mass spectrum of main UV peak of control; (C) ESI mass spectrum of main UV peak of after treatment with 0.03% H₂O₂.

Table 2 Oxidative Stress of MOE Gapmer **1**

<i>m/z</i> (Observed)	Description	Percent of Sample	
		Control	H ₂ O ₂ Treated
1730.5	Parent oligonucleotide (1)	87.7	76.9
1701.3	(P=O) ₁	2.1	11.1
1697.2	(P=O) ₂	ND ^a	0.95
NA	Early eluting components	2.4	4.1

^aNone detected (lower limit of detection = 0.1%).

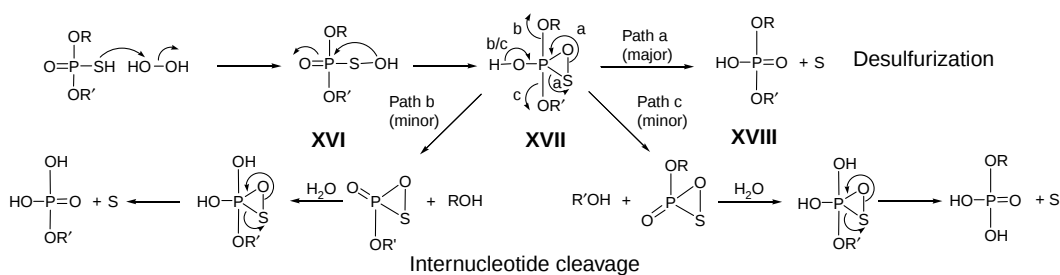


Figure 14 Potential mechanism of desulfurization and internucleotide cleavage of phosphorothioate diesters upon exposure to H_2O_2 .

results in internucleotide cleavage as well as sulfur for oxygen exchange. This is reminiscent of the reactivity of phosphorothioate diesters toward iodoethanol, treatment, which while leading mainly to desulfurization also results in small amounts of internucleotide cleavage and thereby constitutes a method for sequencing DNA (57). A potential mechanism that explains the observed internucleotide cleavage pathway provided in Figure 14 (paths b and c).

The phosphorothioate diester linkages of MOE gapmers are an opportunity for degradation not open to naturally occurring DNA and RNA. Although a direct comparison to literature reports for phosphate diester DNA and RNA is difficult to make, the results obtained with MOE gapmers suggest the latter degrade significantly more rapidly. This suggests that while the base and sugar oxidation pathways described in the literature for phosphate diesters are undoubtedly also open to MOE gapmers, degradation of the phosphorothioate diester linkage, which results mainly in sulfur loss and some internucleotide cleavage, dominates and that the degradation pathways and products described for DNA and RNA will only begin to become important after the parent MOE gapmer has degraded significantly.

THERMAL STRESS

Not surprisingly, a great deal of information is available on the thermal stability of nucleic acids. Among other things, an understanding of the thermal stabilities of DNA and RNA is important to theories of mutagenesis, carcinogenesis, and cellular aging, to the study of ancient genetic material obtained from extinct animals and plants and to those working in the field of prebiotic chemistry. In biological systems, three hydrolytic reactions dominate. In DNA, the main degradation pathways are hydrolysis of the glycosidic bond of 2'-deoxyadenosine and 2'-deoxyguanosine residues (58) and deamination of 2'-deoxycytosine residues (59). In RNA, the presence of the 2'-OH group means that a third hydrolytic pathway, namely internucleotide cleavage and migration, is also important (60).

Adenosine and guanine are lost from DNA at similar rates. Depurination rates are pH sensitive (see section "Acid Stress"). The first-order rate constant of depurination of single stranded DNA is $\sim 1 \times 10^{-10} \text{ s}^{-1}$ at pH 7.4 and 37°C and the rate in double-stranded DNA is about four times slower (58). Depyrimidation, that is, loss of cytosine or thymine, occurs at about 5% the rate of depurination (61). In RNA, the presence of the 2'-OH groups retards the rate of depurination by between two and three orders of magnitude. The products of depurination are a purine heterocycle and a nucleic acid containing an abasic site. The abasic site exists as an epimeric mixture of ring closed isomers **X**, which is in equilibrium with a small amount (ca. 1%) of the corresponding aldehyde tautomer **XIX** (Fig. 15). **XIX** is unstable under physiological conditions and rather rapidly undergoes β -elimination to give two shorter oligonucleotides, **XX** and **XXI** (62). **XX** may participate in a second, slower β -elimination reaction (by the principle of vinylology the indicated hydrogen atom is acidic) giving oligonucleotide **XXII** and 4-oxo-pentenal (2).

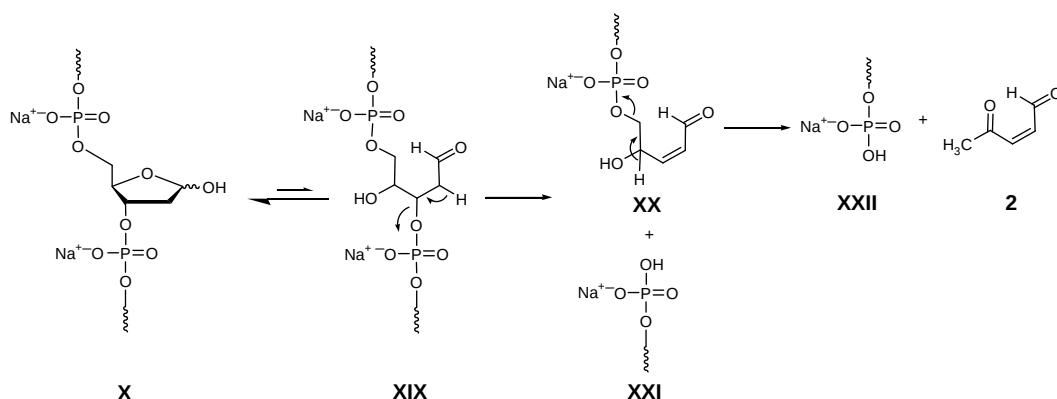


Figure 15 Cleavage of an abasic oligonucleotide.

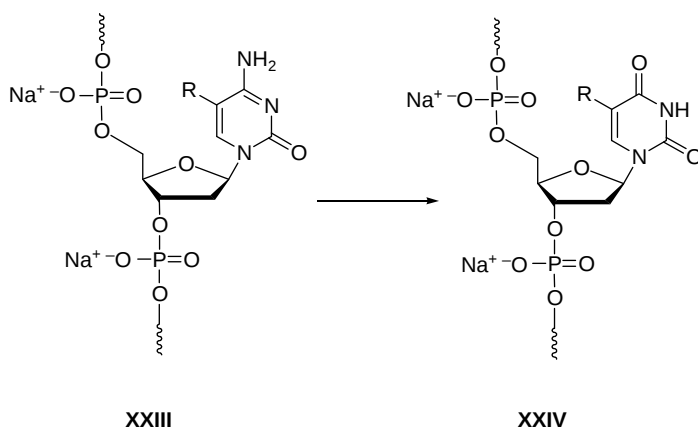


Figure 16 Deamination of cytosine ($R = H$) and 5-methylcytosine ($R = CH_3$) residues in DNA.

The second major hydrolytic degradation pathway entered into by nucleic acids is deamination, which results primarily in conversion of cytosine to uracil (Fig. 16, $R = H$). Deamination of adenine residues also occurs, but at physiological pH, the rate is about 50 times slower than the deamination rate of cytosine (63). The deamination rate of cytosine is constant from pH 0 to 3. From there it decreases up to pH 6, where it remains constant until pH 8 before increasing rapidly at more alkaline pH (64). The deamination rate of cytosine residues in single-stranded DNA is ca. $1 \times 10^{-10} \text{ M s}^{-1}$ at pH 7.4 and 37°C , which is about the same as the depurination rate under the same conditions (59).

Interestingly, the rate of deamination of cytosine residues in double-stranded DNA is more than two orders of magnitude slower than the rate in single-stranded DNA (65). The deamination rate of 5-methylcytosine to thymine (Fig. 16, $R = CH_3$) is approximately five times faster than the rate of conversion of cytosine to uracil (66).

A third major hydrolytic degradation route, which is relevant for RNA only, is the direct hydrolysis of internucleotide linkages. The phosphate diester internucleotide linkages of DNA are actually very resistant to hydrolysis at neutral pH, mainly because they are negatively charged (67). The first-order rate constant for internucleotide cleavage of DNA is ca. $1 \times 10^{-14} \text{ s}^{-1}$ at pH 7.4 and 37°C , which is ca. 10,000 times slower than the rates of depurination and deamination (68). Because the vicinal 2'-OH group acts as an internal nucleophile, the rate of

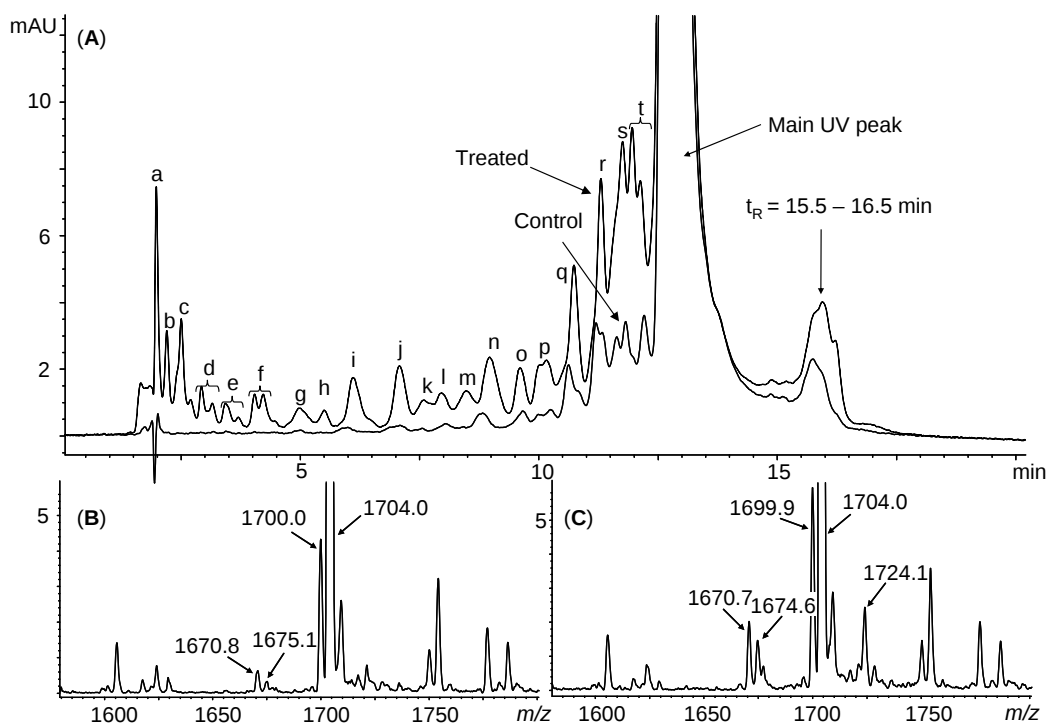


Figure 17 Thermal stress of MOE gapmer 8: (A) IP-HPLC-UV chromatogram; (B) ESI mass spectrum of main UV peak of control; (C) ESI mass spectrum of main UV peak of sample after heating at 80°C for 29 days.

internucleotide cleavage of RNA is much faster. The rate is sequence dependent and greatly accelerated by the presence of divalent cations such as Mg and Ca, but, very roughly, a phosphate diester bond in RNA hydrolyzes about 1 million times faster than one in DNA (69).

We have studied the degradation pathways and products obtained upon exposing MOE gapmer drug substances (lyophilized powders) to conditions of thermal stress. Our findings are largely in line with those described above for phosphate diester DNA. That is, we find that MOE gapmers mostly undergo deamination and depurination and that the abasic oligonucleotides formed by depurination cleave to produce shorter oligonucleotides. Depurinated oligonucleotides also appear to react with molecules of the parent oligonucleotide to give a variety of products. We assessed the impact of thermal stress on drug substance by heating lyophilized MOE gapmer oligonucleotide $\text{G}^{\text{Me}}\text{C} \text{A}^{\text{Me}}\text{CTTTGTGGTG}^{\text{Me}}\text{C}^{\text{Me}}\text{CAAGG}^{\text{Me}}\text{C}$ (3) in a sealed vial at 80°C for 29 days. A control sample was kept at -20°C for the same period. Following incubation, the contents of the sample and control vials were analyzed by IP-HPLC-ESI-MS. The results are shown in Figure 17 (70).

Figure 17(A) clearly shows that thermal stress of MOE gapmer 3 results in an increase in components that elute earlier than the parent oligonucleotide. By analyzing the mass spectra under the peaks marked *a* through *s*, we were able to assign tentative structures to the main components of each peak. These data are provided in Table 3.

The average mass spectra were congruent with oligonucleotides that lack nucleotides from either the 3' or 5'-end of the parent sequence. Terminally thiophosphorylated [appended with -OPO(OH)SH in Table 3] and nonthiophosphorylated (-OH in Table 3) sequences were observed. The majority of -OPO(OH)SH components were sequences consistent with a depurination-elimination-mechanism of formation (Fig. 15). It seems likely that the -OH species arise by dethiophosphorylation (Fig. 18), perhaps via monomeric metathiophosphate (4) (71).

Table 3 Observed Masses, Proposed Structures, and Predicted Most Abundant Molecular Masses for Peaks a Through s of Figure 17(A)

Peak	Observed Mass (amu)	Proposed Structure ^a	Calculated Most Abundant Mass (amu)
a	None	Unknown ^b	N/A
b	1079.6	HO-GG ^{Me} C	1079.3
c	1408.4	HO-AGG ^{Me} C	1408.3
d	1504.4	HS(HO)OPO-AGG ^{Me} C	1504.2
e	2056.8	HO-MeCAAGG ^{Me} C	2056.4
f	2343.2	G ^{Me} CA ^{Me} CTTT-OH	2343.4
	2376.3	HO-MeC ^{Me} CAAGG ^{Me} C	2376.4
g	2472.2	HS(HO)OPO-MeC ^{Me} CAAGG ^{Me} C	2472.3
	2688.5	G ^{Me} CA ^{Me} CTTTG-OH	2688.4
	2721.4	HO-G ^{Me} C ^{Me} CAAGG ^{Me} C	2721.4
h	2439.5	G ^{Me} CA ^{Me} CTTT-OPO(OH)SH	2439.3
	2518.8	Unknown	N/A
i	3008.7	G ^{Me} CA ^{Me} CTTTGT-OH	3008.4
	3041.7	HO-TG ^{Me} C ^{Me} CAAGG ^{Me} C	3041.4
j	3353.7	G ^{Me} CA ^{Me} CTTTGTG-OH	3354.4
	3386.8	HO-GTG ^{Me} C ^{Me} CAAGG ^{Me} C	3387.5
k	3104.7	G ^{Me} CA ^{Me} CTTTGT-OPO(OH)SH	3105.4
	3138.0	HS(HO)OPO-TG ^{Me} C ^{Me} CAAGG ^{Me} C	3138.4
	3185.3	Unknown	N/A
l	3699.5	G ^{Me} CA ^{Me} CTTTGTGG-OH	3699.5
	3732.8	HO-GGTG ^{Me} C ^{Me} CAAGG ^{Me} C	3732.5
m	3450.0	G ^{Me} CA ^{Me} CTTTGTG-OPO(OH)SH	3450.4
	3483.4	HS(HO)OPO-GTG ^{Me} C ^{Me} CAAGG ^{Me} C	3483.4
	3530.3	Unknown	3530.4
n	4019.4	G ^{Me} CA ^{Me} CTTTGTGGT-OH	4019.5
	4053.1	HO-TGGTG ^{Me} C ^{Me} CAAGG ^{Me} C	4052.5
o	4364.8	G ^{Me} CA ^{Me} CTTTGTGGT-OH	4364.5
	4397.3	HO-GTGGTG ^{Me} C ^{Me} CAAGG ^{Me} C	4397.6
p	4115.9	G ^{Me} CA ^{Me} CTTTGTGGT-OPO(OH)SH	4115.4
	4148.2	HS(HO)OPO-TGGTG ^{Me} C ^{Me} CAAGG ^{Me} C	4148.5
	4195.6	Unknown	4195.4
q	5003.3	G ^{Me} CA ^{Me} CTTTGTGGTG ^{Me} C ^{Me} C-OH	5003.6
r	5332.9	G ^{Me} CA ^{Me} CTTTGTGGTG ^{Me} C ^{Me} CA-OH	5332.6
s	5099.4	G ^{Me} CA ^{Me} CTTTGTGGTG ^{Me} C ^{Me} C-OPO(OH)SH	5099.5
	5179.8	Unknown	5179.5
	5662.3	G ^{Me} CA ^{Me} CTTTGTGGTG ^{Me} C ^{Me} CAA-OH	5661.7
t	5428.5	G ^{Me} CA ^{Me} CTTTGTGGTG ^{Me} C ^{Me} CA-OPO(OH)SH	5428.6
	5678.2	HO-MeCTTTGTGGTG ^{Me} C ^{Me} CAAGG ^{Me} C	5677.7

^aUnderlined residues are 2'-MOE, all other residues are 2'-deoxy.^bWe believe peak a is due to adenine and guanine lost when **3** undergoes depurination.

The average mass spectra of the main UV peak of the control and stressed samples (Fig. 18(B) and (C), respectively) were dominated by a signal at $m/z = 1704.0$, which was consistent with the -4 charge state of MOE gapmer **3** (calc. most abundant mass = 1704.0). Close inspection of the average mass spectra revealed four components increased following thermal stress. First, we noted a slight increase in the (P=O)₁ component (Fig. 3) (calc. $m/z = 1700.0$; obs. $m/z = 1699.9$), which suggested a small fraction of molecules suffered desulfurization under these conditions. More obvious were increases in components with m/z values of 1670.7 and

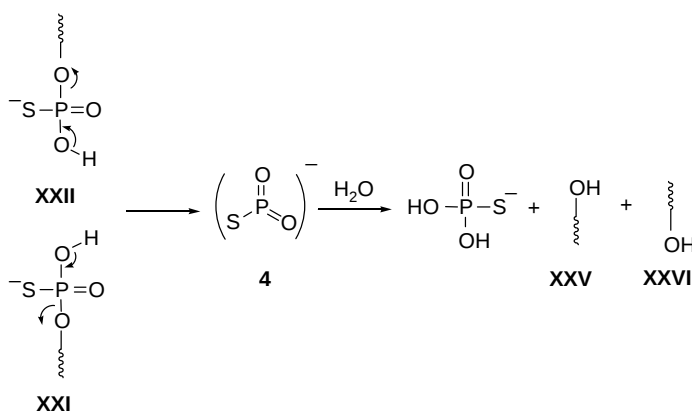


Figure 18 Dethiophosphorylation of thiophosphate monoesters.

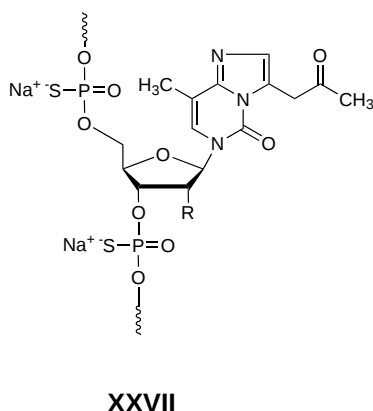


Figure 19 Structure of the $m/z = 1724.1$ component ($R = \text{H}$ or $\text{OCH}_2\text{CH}_2\text{OCH}_3$).

1674.6, which were consistent, respectively, with loss of guanine and adenine from **3** followed by the addition of water (calc. $m/z = 1670.7$ and 1674.4 , respectively). That depurination occurs under conditions of thermal stress is consistent with known degradation pathways of DNA and lends support to the hypothesis that most the products of internucleotide cleavage described in Table 3 arise by the mechanism outlined in Figure 15. Interestingly, thermal stress was also accompanied by the appearance of a component with $m/z = 1724.1$, which was absent from the control sample. We believe this species has the general structure **XXVII** (Fig. 19) (calc. $m/z = 1724.0$) and forms when 5-methylcytosine residues react with 4-oxo-pentanal (**2**, Fig. 15), which is a potential by-product of depurination (37). Presumably any one of the 5-methylcytosine residues of **3** may be converted to a 3-(2-oxopropyl)imidazo(1,2-c)pyrimidin-5-methyl-6H-one residue, implying that **XXVII** could be a mixture of five different species.

Lastly, in regard to the data provided in Figure 17, we observed a slight increase in components that elute after the main UV peak [Fig. 17(A), $t_R = 15.5 - 16.5$ minutes]. The average mass spectrum in this region of the chromatogram of the treated sample is shown in Figure 20.

Several peaks were evident in this region of the chromatogram. The data were consistent with the presence of four different types of component, for which we propose generalized structures **XXVIII**, **XXIX**, and **XXX** (Fig. 21).

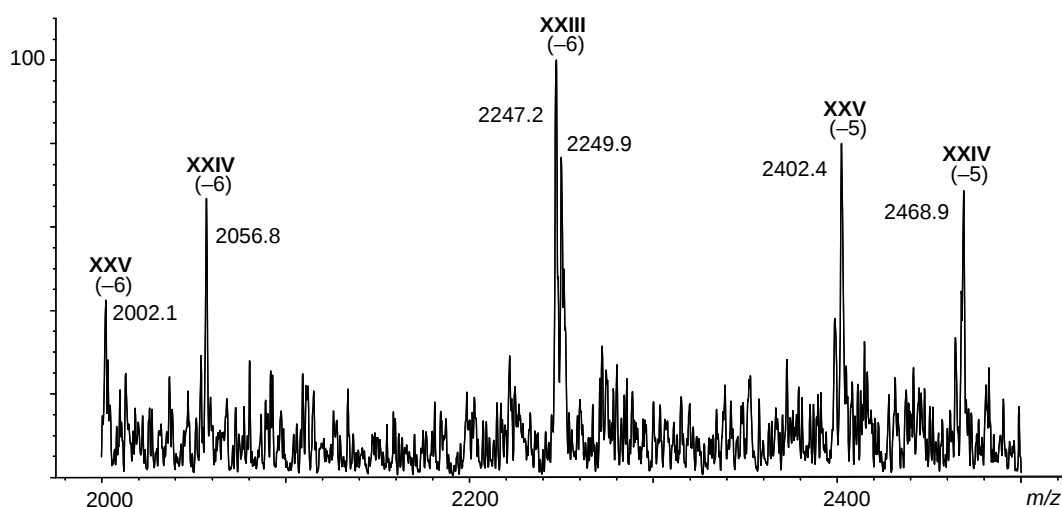
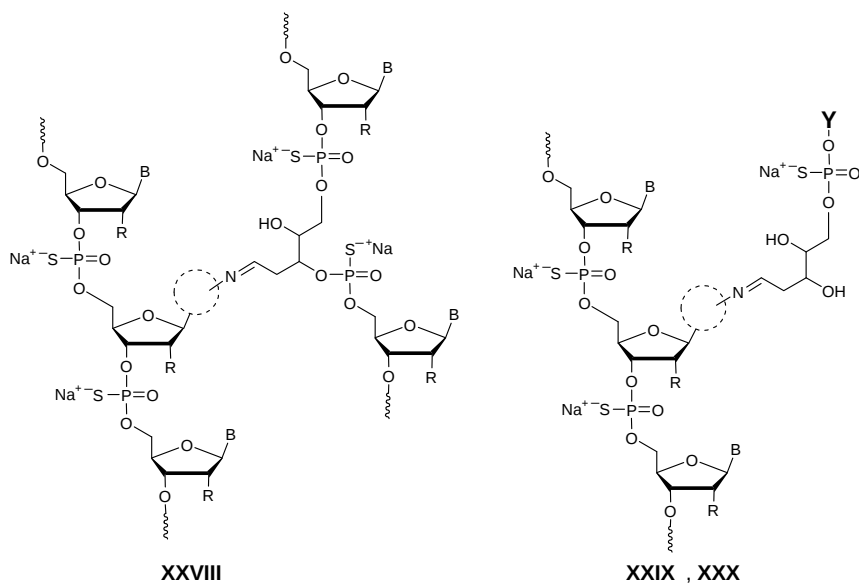


Figure 20 Average mass spectrum of thermally stressed sample between $t_R = 15.5$ and 16.5 minutes (charge states are indicated in parentheses). Proposed structures for **XXVIII**, **XXIX**, and **XXX** are provided in Fig. 21.



Compound	Y	Calc.mass(amu)	Obsd.mass(amu)
XXVIII (-adenine)	NA	13504.6	13505.4
XXVIII (-guanine)	NA	13488.8	13489.2
XXIX	$\begin{array}{c} \text{Me} \\ \text{CA} \end{array} \text{CTTTGTGGTG} \begin{array}{c} \text{Me} \\ \text{C} \end{array} \begin{array}{c} \text{Me} \\ \text{C} \end{array}$	12017.4	12017.8
XXX	$\begin{array}{c} \text{Me} \\ \text{CA} \end{array} \text{CTTTGTGGTG} \begin{array}{c} \text{Me} \\ \text{C} \end{array} \begin{array}{c} \text{Me} \\ \text{CA} \end{array}$	12347.5	12348.2

Figure 21 Structures of $t_R = 15.5 - 16.5$ minutes' components formed by thermal stress of **3**. The dotted circle represents a cytosine or a guanine heterocycle (see the text for discussion).

The two heaviest components had most abundant masses of 13505.4 and 13489.2 Da, which were consistent with general structure **XXVIII**. We believe these species arose by condensation of **3** with molecules that had undergone depurination at 2'-deoxyadenosine and 2'-deoxyguanosine sites, respectively. It should be acknowledged that other than mass agreement, we have no evidence of the nature of the bond linking the two strands together, which is drawn here as a Schiff base formed between an exocyclic amino group of one strand and the abasic site of the second. However, the association between inter-strand cross-linking (ICL) and depurination was recognized some time ago (72,73) and more recently, Gates described ICL in duplex DNA as a result of Schiff base formation through the N^2 atom of guanine (74). Our data describing the reactivity of cytosine residues in the presence of abasic sites suggest that ICL at cytosine residues is also likely. The two lighter components observed had most abundant masses that were consistent with general structures **XXIX** and **XXX**. We suggest these components form by cleavage of the Schiff bases formed between **3** and molecules that have undergone depurination at the first and second 2'-deoxyadenosine residues of **3**, respectively.

It is apparent from the foregoing that depurination of 2'-deoxypurine residues is an important first step in the thermal degradation of MOE gapmer oligonucleotides. Depurination results in oligonucleotides that contain an abasic site, which can react further to give shorter oligonucleotides, oligonucleotides containing a (2-oxopropyl)imidazo(1,2-c)pyrimidin-5-methyl-6H-one residue (Fig. 19) and a variety of cross-linked species (Fig. 21).

In addition to depurination, the other main hydrolytic degradation pathway open to DNA is deamination of cytosine residues to uracil (Fig. 16, R = H). For many oligonucleotides, however, measuring the deamination rate is not straightforward. For example, although chromatographic methods that separate oligonucleotides on the basis of sequence have been developed, we are unaware of reports describing the application of these methods to the quantification of deaminated degradation products. For phosphorothioate diester and other oligonucleotides of undefined internucleotide linkage chirality, where the presence of often several hundred thousand diastereoisomers² results in rather broad chromatographic peak shapes, chromatographic separation of deaminated degradation products is likely to be even more difficult. Additionally, because parent and singly deaminated oligonucleotides are separated by only 1 Da, the mass spectrometry method described in section "Analytical Methodology", which is able to resolve components that differ by as little as 4 Da, cannot be used to determine deamination rates. Clearly mass spectrometers with resolving powers far exceeding that of the single quadrupole instruments used routinely at Isis are widely available. For example, isotopic resolution of MOE gapmer oligonucleotides is easily accomplished using a benchtop, time-of-flight (TOF) instrument. However, because singly deaminated species are 1 amu heavier than the parent, the presence of low concentrations of the former result in only small differences in isotope distribution pattern. Therefore, even when isotopic resolution is achieved, it is difficult to use the isotopic distribution pattern to estimate deamination levels. Of course, the mass defect means the isotope distribution patterns of the parent and deaminated components do not overlap exactly. Unfortunately, the degree of resolution required to take advantage of the difference engendered by the mass defect, which is on the order of several hundreds of thousands, is beyond the capabilities of all but the most powerful ion cyclotron resonance (ICR) mass spectrometers.

We have developed two methods suitable for determining deamination levels in oligonucleotides. In the first method, which works well for first-generation phosphorothioate oligonucleotides composed of the naturally occurring 2'-deoxynucleosides, the sample is first treated with iodine in the presence of wet base, which converts the phosphorothioate diester linkages

²The number of diastereoisomers is equal to 2^n , where n is the number of chiral internucleotide linkages. For example, a 20-mer phosphorothioate oligonucleotide that contains 19 chiral phosphorothioate diester internucleotide linkages comprises 524,288 diastereoisomers.

to phosphate diesters. Once the sulfur atoms have been removed, the oligonucleotide is incubated with a mixture of snake venom phosphodiesterase (SVPDE) and alkaline phosphatase. The resulting nucleoside products are then analyzed by HPLC and the degree of deamination estimated from the ratio of 2'-deoxyuridine to 2'-deoxycytidine.

Two prerequisites must be met in order for this approach to be useful: First, the oligonucleotide should be susceptible to nuclease degradation, or, as is the case with phosphorothioate oligonucleotides, easily converted to a digestible form. Second, the nucleotide composition of parent oligonucleotide should not include the product of deamination. That is, the method is much more difficult to apply to a sequence that contains, for example, 2'-deoxycytidine and 2'-deoxyuridine residues than it is to one that contains 2'-deoxycytidine and thymidine. This is because in the latter, provided appropriate HPLC conditions are developed, deamination at the oligonucleotide level is manifest by the appearance of a peak due to 2'-deoxyuridine in an otherwise empty region of the chromatogram of the enzyme digest, while in the former, because the parent sequence contains 2'-deoxyuridine, deamination results in an increase in a peak already present. When deamination levels are low, it is clearly easier to detect the presence of a new peak in a flat region of the chromatogram than it is a small increase in a relatively large peak.

Unfortunately, the majority of MOE gapmers in development fulfill neither prerequisite. That is, even following desulfurization, MOE gapmers are difficult to digest fully. Additionally, the majority of MOE gapmers are sequences composed of 5-methyl-2'-deoxycytidine and thymidine and 5-methyl-2'-*O*-(2-methoxyethyl)cytidine and 5-methyl-2'-*O*-(2-methoxyethyl)uridine. This means that the products of deamination are, more often than not, present as part of the parent sequence. It was as a result of these challenges that we set out to develop a second method, which we hoped would prove suitable for determining deamination levels in second-generation oligonucleotides (75). The method is described in outline in Figure 22.

In the second approach, which can be thought of as a form of hybridization-protection assay, oligonucleotides complementary to the parent oligonucleotide (parent complement) and each of its singly deaminated degradation products (deaminated complements) are first added to a solution of the sample in a suitable buffer. The complements, which are phosphate diester oligonucleotides that present a short run of 2'-*O*-alkyl nucleotides at their 5'-end, are added in excess of the parent sequence and its deamination products. The mixture of sample and complements is heated above the temperature at which only single-stranded oligonucleotides are present and then allowed to cool to room temperature. During the cooling process, the parent sequence and its deamination products form duplexes with their respective complements. Following the annealing step, excess complements are digested by the addition of nuclease S1. Because nuclease S1 digests only single-stranded oligonucleotides, those complement molecules that are present as one half of a duplex are protected from digestion. Additionally, the single-stranded overhangs formed by the extra nucleotides of the complements, because they are constructed of 2'-*O*-alkyl substituted nucleosides, are also protected from S1-catalyzed hydrolysis. Following S1 treatment, the sample solution, which now contains the various duplexes and a mixture of short oligonucleotides and nucleotides formed by digestion of excess complements, is analyzed by HPLC under denaturing conditions. The resulting chromatogram comprises three distinct regions. The early part of the chromatogram is dominated by nucleotides and short oligonucleotides that are formed by digestion of excess complements. Two additional peaks are observed. The first peak is due to the parent sequence and any deamination products thereof, while the second comprises those complement molecules that bound to the parent sequence and its deamination products during the annealing step. Because they are modified by a unique string of nucleotides attached to their 5'-ends, each complement has a different mass. Extraction of the mass signals due to each complement and subsequent integration of the resulting mass chromatograms allows one to determine the ratios of each deamination complement to the parent complement. Provided only perfect duplexes formed in the annealing step, the ratio of complements in the second peak reflects the ratio of parent oligonucleotide and deamination products in the first peak.

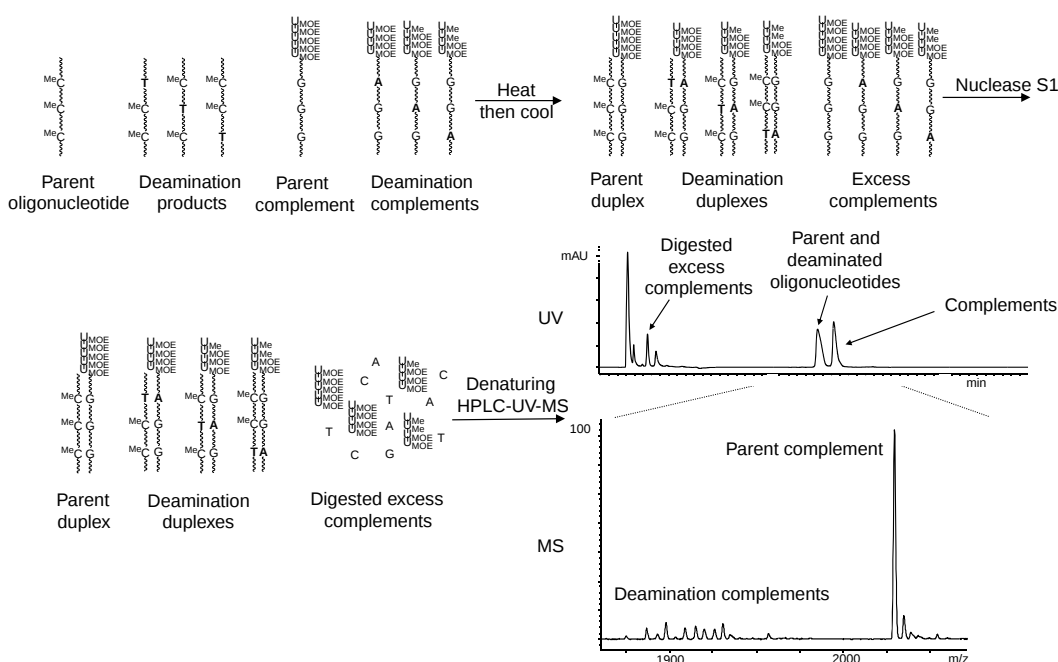


Figure 22 Method for determining deamination rates of MOE gapmers (see the text for explanation).

To test the idea, we synthesized a number of singly deaminated components of a MOE gapmer oligonucleotide and used these materials to prepare a series of samples containing known amounts of deamination. To each sample was added a fixed amount of a cocktail containing the parent complement and complements to each deaminated species added. The samples were treated as described above and for each deamination species, the ratio of complements measured (deamination:parent) was plotted against the ratio of deaminated to parent component expected. An example recovery plot is reproduced in Figure 23.

The approach was used to determine deamination rates for eight of the nine 5-methylcytosine residues of MOE gapmer $\underline{\text{C}}^{\text{Me}}\underline{\text{C}}^{\text{Me}}\underline{\text{C}}^{\text{Me}}\underline{\text{U}}^{\text{Me}}\underline{\text{C}}\underline{\text{A}}\underline{\text{G}}\underline{\text{T}}^{\text{Me}}\underline{\text{C}}\underline{\text{T}}\underline{\text{G}}^{\text{Me}}\underline{\text{C}}\underline{\text{T}}\underline{\text{T}}^{\text{Me}}\underline{\text{C}}\underline{\text{G}}^{\text{Me}}\underline{\text{C}}\underline{\text{A}}^{\text{Me}}\underline{\text{C}}^{\text{Me}}\underline{\text{C}}^{\text{Me}}$ (5).³

Lyophilized drug substance and drug product formulated at 200 mg/mL in aqueous solution at pH 7.7 were heated at 80°C for 2 weeks and for 4 days, respectively. The results of the analysis are shown in Table 4 (76).

For lyophilized drug substance, the rate of deamination at 80°C was too slow to be estimated. For the solution drug product, however, deamination occurred at a measurable rate at all sites. There was an approximately twofold difference in deamination rates between the slowest and fastest reacting sites. The average deamination rate was ca. $7 \times 10^{-8} \text{ M s}^{-1}$, which

³The method is reliant on the selective formation of parent species:parent complement and deaminated species: deamination complement duplexes. Selectivity is predicated on the relative stabilities of the parent duplexes and their mismatched pairs. When the mismatch is located at the 3' end of oligonucleotide 5, the stability difference between perfect and mismatched duplexes is insufficient to favor formation of perfect duplexes over imperfect ones. In this case, the deamination:parent complement ratio does not increase as deaminated species is added and the recovery graph is a horizontal line with a y-intercept that approximates the ratio of parent and deaminated complements added to the sample.

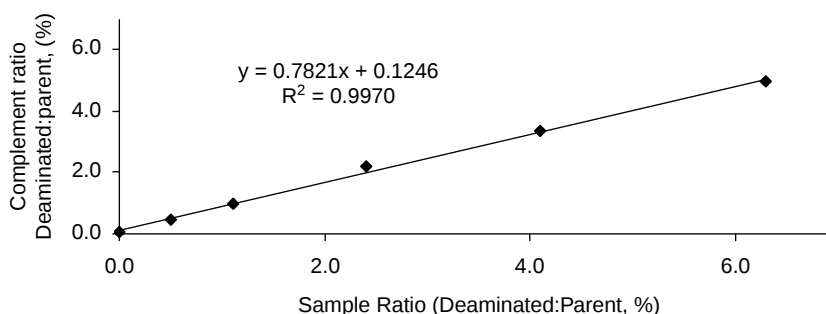


Figure 23 Complement ratio against deamination:parent species ratio.

Table 4 Solid and Solution Deamination Rate Constants of MOE Gapmer **5** at 80°C

Deamination Site ^a	First-Order Deamination Rate Constant at 80°C (s ⁻¹)	
	Lyophilized Drug Substance	Solution Drug Product ^b
1	$< 5 \times 10^{-9}$	4×10^{-8}
2	$< 5 \times 10^{-9}$	5×10^{-8}
3	$< 5 \times 10^{-9}$	8×10^{-8}
4	$< 5 \times 10^{-9}$	1×10^{-7}
5	$< 5 \times 10^{-9}$	9×10^{-8}
6	$< 5 \times 10^{-9}$	1×10^{-7}
7	$< 5 \times 10^{-9}$	4×10^{-8}
8	$< 5 \times 10^{-9}$	6×10^{-8}

^aMethylcytosine residue from the 5'-end of **5**.

^b200 mg in water, pH 7.5.

was similar to the rate of $8 \times 10^{-8} \text{ M s}^{-1}$ reported for a solution of cytidine held at pH 7.5 and 80°C (64).

BASIC STRESS

DNA is more stable at high pH than at low pH. However, under forcing basic conditions, DNA degrades to a variety of products. As was alluded to in section "Thermal Stress", the rate of deamination of cytosine nucleosides increases rapidly with increasing pH. For example, at 80°C, the rate of deamination of cytidine increase from ca. $8 \times 10^{-8} \text{ M s}^{-1}$ at pH 7.5 to ca. $1 \times 10^{-5} \text{ M s}^{-1}$ at pH 11.5 to ca. $1 \times 10^{-4} \text{ M s}^{-1}$ at pH 12.3 (64). At 70°C, the rate of deamination of cytosine residues in DNA in 1 M NaOH is about $1 \times 10^{-5} \text{ M s}^{-1}$, which is about half the rate of deamination of 2'-deoxycytidine (77).

Although cytidine deamination (6→7) occurs at all pH values, a competing degradation pathway, which results in irreversible ring opening of the heterocyclic ring system (6→9), is also important above pH 10 [Fig. 24 (64)].

Thymine nucleosides are more stable toward alkaline hydrolysis than cytosine nucleosides. For example, it was reported that 5-methyluridine is unchanged after heating in 0.4 N NaOH for 5 days at 80°C (78).

The alkaline hydrolysis of adenine nucleosides was studied in detail by Garrett (79). In 1.0 N NaOH, degradation was shown to proceed via two parallel routes (Fig. 25).

For example, incubation of a 1.0 N NaOH solution of 2'-deoxyadenosine (**10**) at 80°C results in hydrolysis of the glycosidic bond to give adenine (**11**) at a rate of $2.2 \times 10^{-5} \text{ s}^{-1}$.

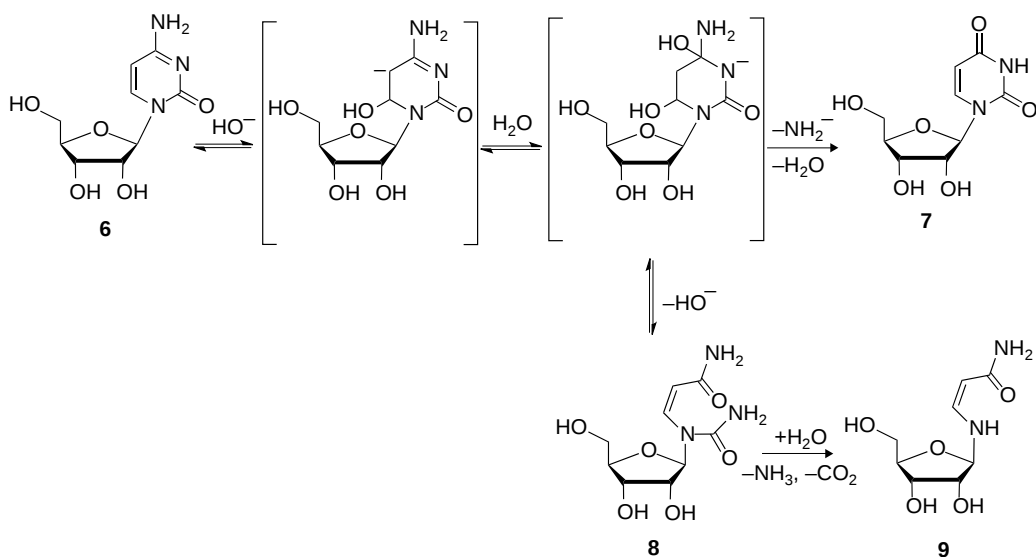


Figure 24 At high pH (>10) deamination cytidine (6→7) competes with ring opening of the heterocyclic ring system (6→9).

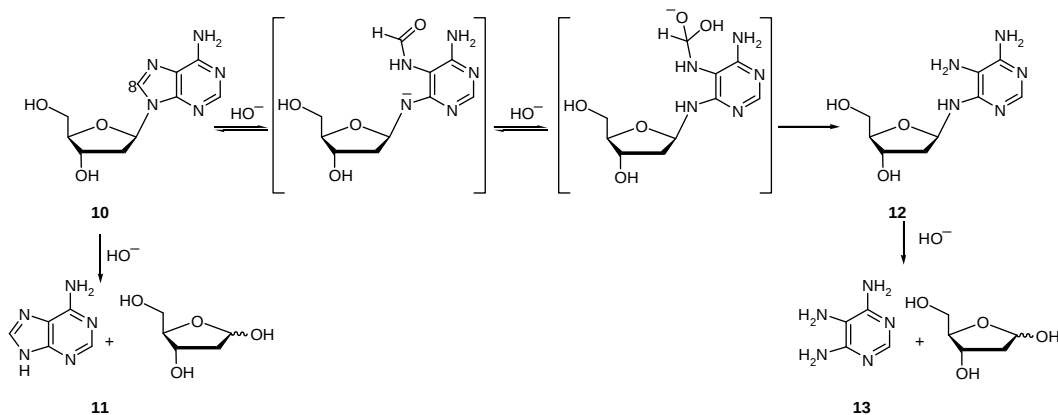


Figure 25 Alkaline hydrolysis of 2'-deoxyadenosine.

Concurrently, hydroxide attacks C8, which is subsequently lost as formic acid, giving 4,5,6-triaminopyrimidine nucleoside **12** (rate = $3.8 \times 10^{-5} \text{ M s}^{-1}$). Under these conditions, the glycosidic bond of **12** cleaves relatively rapidly (rate = ca. $2.5 \times 10^{-4} \text{ M s}^{-1}$) to give 4,5,6-triaminopyrimidine (**13**). Guanine nucleosides are considerably more stable under alkaline conditions than adenine nucleosides, presumably because deprotonation of N^1 renders them less susceptible to nucleophilic attack (80).

At the DNA level, the reactions described in Figure 25 would be expected to result initially in the formation of oligonucleotides that contain abasic sites. It would also be anticipated that under the conditions of their formation, the initially formed products would immediately cleave to shorter fragments. This expectation is supported empirically and incubation of a solution of DNA in 1.0 M NaOH at 70°C results in chain cleavage at a rate of $\sim 1/10$ th the rate of cytosine deamination (79).

Table 5 Deamination Rate Constants of MOE Gapmer **5** in 1 N NaOH

Deamination Site ^a	First-Order Deamination Rate Constant (s ⁻¹)
1	3×10^{-7}
2	2×10^{-7}
3	3×10^{-7}
4	1×10^{-7}
5	2×10^{-7}
6	2×10^{-7}
7	3×10^{-7}
8	5×10^{-8}

^aMethylcytosine residue from the 5'-end of **5**.

In contrast to DNA, which is relatively stable at high pH, RNA, by virtue of its 2'-OH group, is unstable in alkaline conditions. It has been estimated that the half life-time of an RNA linkage at pH 13 and 23°C is ~ 1 hour (81).

We have investigated the degradation products obtained upon exposing MOE gapmer oligonucleotides to alkali. In what was a typical experiment, oligonucleotide **5** (section "Thermal Stress") was dissolved in 1 N NaOH and the resulting solution held at 25°C for 48 hours. Analysis of the stressed sample by IP-HPLC-UV-MS suggested that, apart from a small (<0.5%) increase in earlier eluting components, which we attributed the depurination-cleavage mechanism described above, oligonucleotide **5** was rather stable toward alkaline hydrolysis [data not shown (82)]. However, as was stated above, the IP-HPLC-UV-MS method cannot be used to quantify deamination of 5-methylcytosine residues. Therefore, to investigate the potential for deamination, the same sample of **5** was analyzed using the hybridization-protection assay described in section "Thermal Stress". The results of the analysis are provided in Table 5 (83).

With the exception of position 8, the rate of deamination of **5** was between 1 and 3×10^{-7} M s⁻¹ per 5-methylcytosine residue. The rate measured in this experiment, which was conducted at 25°C, appears to be reasonably consistent with the rate reported at 70°C for deamination of cytosine residues in DNA [1×10^{-5} M s⁻¹ (77)].⁴

PHOTOLYTIC STRESS

Exposure to sunlight is the major determinant in the development of most human skin cancers. The genotoxic potential of sunlight is mostly explained by effects of long-wavelength UVB (295–320 nm) and UVA (320–400 nm) radiation on the nucleobases of DNA.⁵ A comprehensive review of the myriad photoproducts formed upon exposure of nucleic acids to sunlight is beyond the scope of this chapter. In the next few paragraphs, therefore, I will limit my remarks to the main photoproducts that form due to long-wave-length UVB and UVA irradiation oligonucleotides. For a more complete discussion of the products and consequences of

⁴At pH 11, the activation energy (E_A) for deamination of cytidine is ca. 74 kJ/mol (64). Using this value and the rate reported at 70°C for DNA [1×10^{-5} M s⁻¹ (77)], it can be estimated that rate of deamination of DNA at 25°C should be ca. 2×10^{-7} M s⁻¹ in 1 N NaOH, which is reasonably consistent with the rates measured for the majority of cytosine residues of MOE gapmer **5**.

⁵Because they are almost completely blocked by the atmosphere, the direct effects of shortwave UVB (280–295 nm) and UVC (190–280 nm) radiation on DNA, while pronounced, are of no biological consequence.

exposure of DNA to UVA and UVB radiation, the reader may wish to consult one of several recent reviews (84–86).

At dipyrimidine sites, the direct absorption of solar photons in the long-wavelength UVB region of the spectrum mostly results in cyclobutane pyrimidine dimers (CPD, **XXXI**, Fig. 26), which form by (2+2) cycloaddition reaction of the C₅–C₆ double bonds of adjacent thymine or cytosine heterocycles (87). For steric reasons, *syn* diastereoisomers, where the C₅ and C₆ atoms of adjacent heterocycles are linked, are formed selectively over their *trans* counterparts, although the *syn* preference is apparently much stronger in double-stranded oligonucleotides than it is in single-stranded oligonucleotides (88). In addition to CPD, long-wavelength UVB irradiation promotes Paterno–Buchi photoreaction of the C₅–C₆ double bond of one pyrimidine residue with the C₄ C=O or C=NH bond of its neighbor (89). This results in formation of an unstable oxetane or azetidine species **XXXII**, which rearranges to a pyrimidine (6-4)pyrimidine photoproduct **XXXIII** and its related Dewar isomer **XXXIV** (90). A third direct effect of UVB irradiation is the formation of cytosine hydrate **XXXV**, which may then undergo deamination to the corresponding uracil derivative **XXXVI** (91). The major, direct effects of UVB irradiation on DNA are summarized in Figure 26.

CPD are also formed upon exposure of DNA to UVA radiation, although the quantum yield is lower. As DNA nucleobases do not absorb significantly in the UVA region of the electromagnetic spectrum, it has long been accepted that UVA-induced CPD formation must be mediated by one or more photosensitizers, which serve to transfer energy to DNA. Recently, however, Marszalek and co-workers have challenged this view, providing evidence that UVA irradiation of a solution of DNA in pure water results in the formation of CPD and abasic sites (92). Notwithstanding the controversy surrounding the mechanism of UVA-induced CPD formation, it is clear that many of the mutagenic effects of UVA exposure proceed by indirect, photosensitized events. In this regard, products congruent with Type I and Type II processes (93), where the excited sensitizer reacts, respectively, with the substrate, either by hydrogen atom or electron transfer to produce radicals, which subsequently react with oxygen (Type I) or by reaction with dissolved molecular oxygen to give singlet molecular oxygen (¹O₂) (Type II), which then reacts with the substrate, have been described. The precise nature and extent of the products formed by Type I and Type II photosensitized reactions continues to be the subject of vigorous debate. It is likely that much of the disagreement arises because the product mixtures formed appear to be exquisitely sensitive toward the structure of the substrate, that is, nucleoside versus single-stranded oligonucleotide versus double-stranded oligonucleotide and toward the reaction conditions used. However, among the four common nucleobases, it is clear

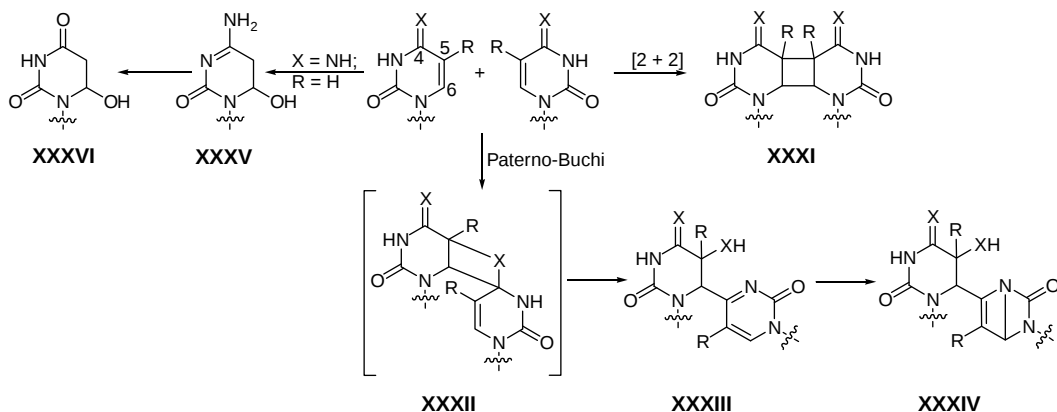


Figure 26 Major photoproducts formed upon exposure of DNA to long-wavelength UVB radiation (when X = O, R = CH₃; when X = NH, R = H).

that guanine is the most susceptible to both one electron oxidation and $^1\text{O}_2$ attack (94). It is also evident that 8-oxo-7,8-dihydroguanine residues (XXXVII, Fig. 27), which, in addition to being a major product of one electron oxidation and $^1\text{O}_2$ attack on guanine, are a ubiquitous marker of oxidative stress (see section "Acid Stress") and are more susceptible to attack by $^1\text{O}_2$ than are guanine residues (95–98). The major products of one electron oxidation of guanine residues and $^1\text{O}_2$ attack on guanine and 8-oxo-7,8-dihydroguanine residues are shown in Figure 27.

Photostress testing of MOE gapmer drug substances and aqueous drug product solutions has been conducted. In what was a typical experiment, MOE gapmer **5** ($\text{C}^{\text{Me}}\text{C}^{\text{Me}}\text{C}^{\text{Me}}\text{U}^{\text{Me}}\text{CAGT}^{\text{Me}}\text{CTG}^{\text{Me}}\text{CTT}^{\text{Me}}\text{CG}^{\text{Me}}\text{CA}^{\text{Me}}\text{C}^{\text{Me}}\text{C}$, section "Thermal Stress") was exposed as a solid in a thin layer and as a 200 mg/mL aqueous solution in a clear glass vial. Dark control samples (samples wrapped in aluminum foil) were placed next to the uncovered samples to control for the effects of heating during the exposure period. The samples were exposed to a total illumination of ca. 27 million lux hours and $\sim 10,000 \text{ W hr/m}^2$ of UVA (ICH Q1B, option 2), or ~ 20 and 50 times the ICH recommended confirmatory levels, respectively. The samples were analyzed by IP-HPLC-ESI-MS. The results obtained when **5** was exposed as a solid are shown in Figure 28 (99).

The most obvious difference between the UV chromatograms of the exposed sample and the dark control was the appearance of a later eluting peak ($t_R = 16.3$ minutes) following irradiation [Fig. 28(A)]. While less striking, it is apparent that irradiation also resulted in an increase in earlier eluting components. MS analysis of the main UV peak [Fig. 28(B) and (C)] showed a significant increase in the monophosphate diester analog of **5** ($m/z = 1789.0$) occurred following exposure. In fact, under these conditions, sulfur loss proceeded to an extent sufficient so that a small fraction of **5** had undergone two desulfurization events, resulting in molecules that contained 17 phosphorothioate diester and two phosphate diester linkages ($m/z = 1784.9$). The observed increase in early eluting species and loss of sulfur were reminiscent of the results obtained upon treatment of MOE gapmers with H_2O_2 (section "Oxidative Stress") and consistent with the intermediacy of ROS; that is, these degradation products are evidence of the indirect effects of UVA irradiation of MOE gapmer drug substance. Evidence of direct photochemical reaction was obtained by analyzing the mass spectrum under the $t_R = 16.3$ minutes peak in the UV chromatogram of the exposed sample. The mass spectrum [Fig. 28(D)] was dominated by two signals at $m/z = 2391.1$ and 2869.4, which were consistent with the -6 and -5

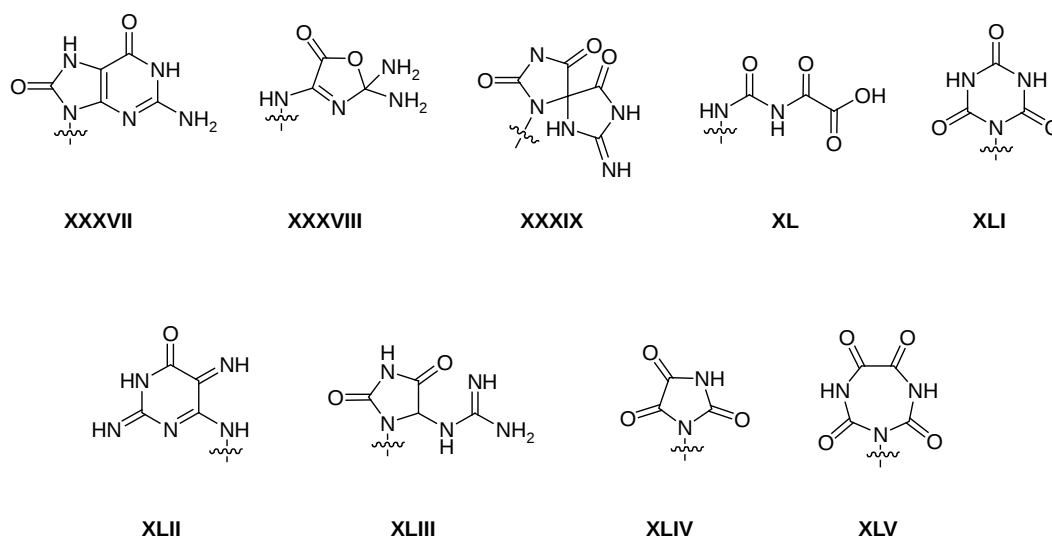


Figure 27 Major products of one electron oxidation and $^1\text{O}_2$ attack on guanine residues.

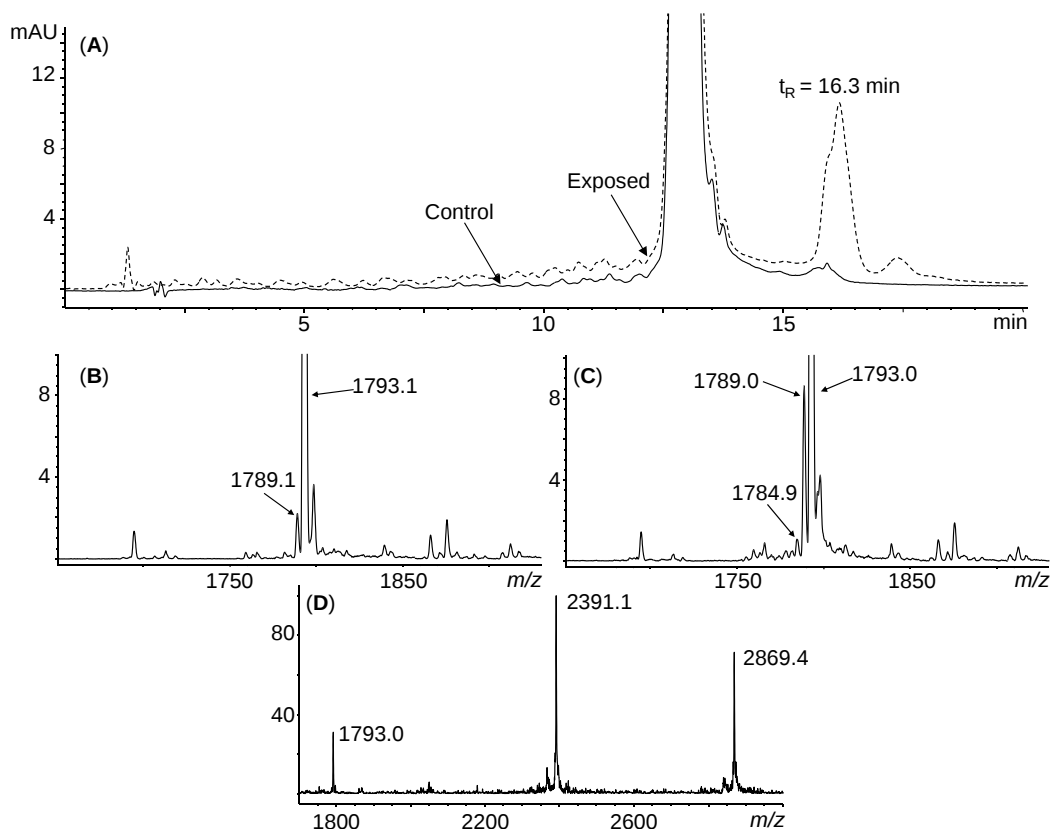


Figure 28 IP-HPLC-UV-MS analysis of **5** exposed to UVA and cool white light as a solid: (A) IP-HPLC-UV trace of dark control (solid line) and exposed drug substance (dashed line); (B) average mass spectrum under main UV peak of dark control; (C) average mass spectrum under main UV peak of exposed sample; (D) average mass spectrum under $t_R = 16.3$ minutes peak of exposed sample.

charge states, respectively, of a component with a most abundant mass of ca. 14352.3 (the signal at $m/z = 1793.0$ was due to the presence of **5**, which tails into this region of the chromatogram). The observed mass was twice the mass of **5** (calc. most abundant mass for **5** = 7176.1), which suggests the $t_R = 16.3$ minutes peak is due to the presence of a photodimer of **5**. We speculate that this component forms by intermolecular 2+2 photocycloaddition reaction of pyrimidine ring systems, which results in molecules that consist of two single strands fused together by a cyclobutane pyrimidine dimer, e.g., **XXXI** (Fig. 26).

As indicated above, oligonucleotide **5** was also irradiated as a 200 mg/mL aqueous solution under the same conditions. The IP-HPLC-ESI-MS results obtained from the dark control and irradiated solution samples are shown in Figure 29 (99).

Comparison of the UV chromatograms of the control and exposed samples indicated that irradiation of an aqueous solution of **5** resulted in cleavage of some fraction of internucleotide linkages [Fig. 29(A)]. Internucleotide cleavage proceeded more readily in solution than in the solid state [cf. Fig. 28(A) with Fig. 29(A)]. Also evident in the UV chromatogram of the exposed sample was a later eluting peak ($t_R = 16.8$ minutes) that was absent from the dark control. The analysis of this component was hampered by a process impurity that was present in this batch of drug substance and that partially co-eluted with the $t_R = 16.8$ minutes' photo degradation product. Interestingly, the average mass spectrum of the $t_R = 16.8$ minutes peak [Fig. 29(D)] was

In contrast to **5**, when a solution of **14** was irradiated under the same conditions there was no evidence of depurination [data not shown, (100)]. This result clearly suggests the $m/z = 1759.8$ signal shown in Figure 29(C) is due to loss of guanine followed by addition of water rather than to loss of adenine followed by addition of a proton. The mechanism by which **5** loses guanine is uncertain. For example, while it is clear that the hydrogen atoms of the sugar portion of DNA may be abstracted by oxidizing agents and free radicals, little if any selectivity among the nucleosides is observed. Furthermore, abstraction of hydrogen atoms normally results directly in strand breakage rather than formation of full-length components that contain abasic sites (53).⁶ It is tempting to speculate that the selective loss of guanine from **5** is due to an initial oxidative attack at the base portion, which leads to products that are prone to glycosidic bond cleavage.

In addition to changes in signals at $m/z = 1789.0$ and $1755.7/1759.8$, which were consistent with loss of sulfur and guanine, respectively, the average mass spectrum of the irradiated solution of **5** showed the appearance of peaks at $m/z = 1801.1$ and 1788.3 , although the latter was mainly obscured by the increase in the peak at $m/z = 1789.0$ due to the $(P=O)_1$ analog of **5**. The structures of these components, which are 32 amu heavier (+32 amu) and 19 amu lighter (−19 amu) than **5**, respectively, have not been elucidated. Interestingly, however, when a solution of **14** was irradiated under the same conditions, neither component was observed [data not shown (100)], which again implicates the involvement guanine. While formal structural proof is lacking for either component, the observed masses of the +32 amu and −19 amu components are consistent with oligonucleotides related to **5** by replacement of a guanine nucleobase with spiroiminodihydantoin **XXXIX** (Fig. 27; calc. $m/z = 1801.0$) and oxaluric acid **XL** (Fig. 27, calc. $m/z = 1788.3$), which have been suggested as major final degradation products formed by photo-oxidation of guanine residues.

SUMMARY

The main degradation products formed when MOE gapmer oligonucleotides are exposed to a variety of chemical and physical insults have been elucidated. Exposure to low pH resulted almost exclusively in depurination, giving oligonucleotides that contain an abasic site at positions formerly occupied by 2'-deoxyadenosine (dA) and 2'-deoxyguanosine (dG). The presence of an electron-withdrawing oxygen atom at the 2'-position stabilizes the glycosidic bond of MOE purine residues toward acid catalyzed cleavage by at least two orders of magnitude relative to dA and dG. Exposure to high pH resulted mainly in deamination of 5-methylcytosine residues to thymine. Oxidative stress resulted mainly in loss of sulfur from phosphorothioate diester linkages. Sulfur loss was accompanied by a small, but reproducible level of internucleotide bond cleavage. The phosphorothioate diester linkages of MOE gapmers clearly render them more susceptible to oxidative stress than naturally occurring phosphate diester DNA.

In contrast to acid, base, and oxidative stress, which caused MOE gapmers to participate in one main degradation pathway to give essentially one type of degradation product, thermal and photolytic stress resulted in more complex mixtures of degradation products. Two main pathways operate under conditions of thermal stress. First, the dA and dG residues of MOE gapmers are subject to thermal depurination. Under the conditions of their formation, the initially formed apurinic species are unstable and cleave to give a variety of shorter oligonucleotides (Figs. 15 and 18). Alternatively, an apurinic oligonucleotide may react with a molecule of the parent, resulting in a family of cross-linked species (Fig. 21). It seems likely that these products are cross-linked via an imine bond, which forms by condensation of the exocyclic amino

⁶A notable exception appears to be H_1' of DNA residues, whose abstraction leads to intact oligonucleotide containing a 2-deoxyribonolactone residue. The calculated m/z value for the 2-deoxyribonolactone analog of **5** is 1759.3, which is inconsistent with the mass observed ($m/z = 1759.8$).

group of guanine and cytosine residues with the aldehyde tautomer of the apurinic site. Finally, depurination also leads to the formation of oligonucleotides that contain a 3-(2-oxopropyl)imidazo(1,2-c)pyrimidin-5-methyl-6H-one residue (Fig. 19) in place of 5-methylcytosine. The second main thermal degradation pathway is deamination of 5-methylcytosine residues to thymine (Fig. 16). Perhaps unsurprisingly, the thermal stability of MOE gapmers is greater in the solid state than it is in solution.

Photolytic stress of solid drug substance resulted mainly in the formation of a family of components of mass exactly twice that of the parent molecule. It seems likely these components arise by photocycloaddition of two molecules of the parent sequence (Fig. 26); their presence, therefore, is evidence of the direct photochemical reaction of DNA nucleobases. In addition, solid state irradiation resulted in sulfur loss and a small amount of internucleotide cleavage, degradation pathways that also operate when MOE gapmers are stressed oxidatively. Degradation via these pathways is evidence of the indirect effect of UVA irradiation, which is mediated through the formation of reactive oxygen species (ROS). While photodimers were also observed following irradiation of an aqueous solution of MOE gapmers, the majority of products formed in solution were consonant with the indirect effects of UVA exposure. Interestingly, in contrast to the indirect effects observed in the solid state, which were limited to internucleotide cleavage and loss of sulfur, those observed in solution were expanded to include attack at guanine residues. The apparent attack of ROS upon guanine, which, of the four common nucleobases is clearly the most susceptible toward such species, resulted in the formation of oligonucleotides containing two different types of modified bases (potentially XXXIX and XL, Fig. 27) and, to a lesser extent, oligonucleotides containing an apurinic site. More work needs to be conducted before the structures of the guanine modifications can be described with confidence.

ACKNOWLEDGMENTS

The author is very grateful to all members past and present of the Analytical Development and Quality Control, Drug Substance Manufacturing and Process Organic Chemistry departments at Isis Pharmaceuticals, Inc.; it is only through their dedication and enthusiasm that so much is known about the degradation pathways and products of phosphorothioate oligonucleotides. He also wishes to acknowledge Stephen Sofen of Genzyme Corporation.

REFERENCES

1. Zamecnik, PC, Stephenson ML. Inhibition of rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. *Proc Natl Acad Sci USA* 1978; 75: 280–4.
2. Sazani P, Graziewicz MA, Kole R. Splice switching oligonucleotides as potential therapeutics. In: Crooke ST, ed. *Antisense Drug Technology: Principals, Strategies and Applications*, 2nd edn. Boca Raton, FL: CRC Press, 2006: 89–114.
3. Baker BF. The 5'-cap of mRNA: biosynthesis, function and structure as related to antisense drugs. In: Crooke ST, Lebleu B, eds. *Antisense Research and Applications*. Boca Raton, FL: CRC Press, 1993: 37–53.
4. Vickers TA, Wyatt JR, Burckin T, Bennett CF, Freier SM. Fully modified 2'-MOE oligonucleotides redirect polyadenylation. *Nucleic Acids Res* 2001; 29: 1293–9.
5. For a review of potential noncoding RNA targets see: Royo H, Bortolin ML, Seitz H, Cavaille J. Small non-coding RNAs and genomic imprinting. *Cytogenet. Genome Res* 2006; 113: 99–108.
6. Vollmer J, Krieg AM. Mechanisms and therapeutic applications of immune modulatory oligodeoxynucleotide and oligoribonucleotide ligands for toll-like receptors. In: Crooke ST, ed. *Antisense Drug Technology: Principals, Strategies and Applications*, 2nd edn. Boca Raton, FL: CRC Press, 2006: 747–72.
7. Wilson C. Aptamer opportunities and challenges. In: Crooke ST, ed. *Antisense Drug Technology: Principals, Strategies and Applications*. 2nd edn. Boca Raton, FL: CRC Press, 2006: 773–99.
8. Herdewijn P. Heterocyclic modifications of oligonucleotides and antisense technology. *Antisense Nucleic Acid Drug Dev* 2000; 10: 297–310.

9. Swayze E, Balkrishen B. The medicinal chemistry of oligonucleotides. In: Crooke ST, ed. *Antisense Drug Technology: Principals, Strategies and Applications*, 2nd edn. Boca Raton, FL: CRC Press, 2006: 143–82.
10. Manoharan M. Oligonucleotide conjugates as potential antisense drugs with improved uptake, biodistribution, targeted delivery and mechanism of action. *Antisense Nucleic Acid Drug Dev* 2002; 12: 103–28.
11. de Fougerolles A, Vornlocher H-P, Maraganore J, Lieberman J. Interfering with disease: a progress report on siRNA-based therapeutics. *Nature Rev Drug Discov* 2007; 6: 443–53.
12. The pharmacology of 2'-deoxyphosphorothioate oligonucleotide has been reviewed: Bennett CF. General pharmacology of phosphorothioate oligodeoxynucleotides. In: Crooke ST, ed. *Antisense Drug Technology: Principals, Strategies and Applications*. New York: Marcel Dekker, 2001: 291–318.
13. Lima W, Wu H, Crooke ST. The RNase H mechanism. In: Crooke ST, ed. *Antisense Drug Technology: Principals, Strategies and Applications*. 2nd edn. Boca Raton, FL: CRC Press, 2006: 47–74.
14. Geary RS, Leeds JM, Fitchett J, et al. Pharmacokinetics and metabolism in mice of a phosphorothioate oligonucleotide antisense inhibitor of *C-raf-1* kinase expression. *Drug Metab Dispos* 1997; 25: 1272–81.
15. Freier SM, Altmann KH. The ups and downs of nucleic acid duplex stability: Structure-stability studies on chemically-modified DNA:RNA duplexes. *Nucleic Acids Res* 1997; 25: 4429–43.
16. Tissue half-life times of MOE oligonucleotides of between 10 and 30 days have been reported. Geary RS, Watanabe TA, Truong L, Freier S, Lesnik EA, Sioufi NB, Sasmor H, Manoharan M, Levin AA. Pharmacokinetic properties of 2'-O-(2-methoxyethyl)-modified oligonucleotide analogs in rats. *J Pharm Exp Ther* 2001; 296: 890–7.
17. Bennett CF. Pharmacological Properties of 2'-O-methoxyethyl modified oligonucleotides. In: Crooke ST, ed. *Antisense Drug Technology: Principals, Strategies and Applications*, 2nd edn. Boca Raton, FL: CRC Press, 2006: 273–303.
18. Henry S, Stecker K, Brooks D, et al. Chemically modified oligonucleotides exhibit decreased immune stimulation in mice. *J Pharm Exp Ther* 2000; 292: 468–79.
19. Nicklin PL, Ambler J, Mitchelson A, et al. Preclinical profiling of modified oligonucleotides: anticoagulation and pharmacokinetic properties. *Nucleosides Nucleotides* 1997; 16: 1145–53.
20. 2'-O-(2-methoxyethyl)phosphorothioate gapmer oligonucleotides are currently being investigated for the treatment of cardiovascular, metabolic and neurodegenerative disorders and in a variety of cancers and inflammatory indications.
21. Capaldi DC, Scozzari AN. Manufacturing and analytical processes for 2'-O-(2-methoxyethyl)-modified oligonucleotides. In: Crooke ST, ed. *Antisense Drug Technology: Principals, Strategies and Applications*. 2nd edn. Boca Raton, FL: CRC Press, 2006: 401–34.
22. Cohen AS, Vilenchik M, Dudley JL, Gemborys MW, Bourque AJ. High-performance liquid chromatography and capillary gel electrophoresis as applied to antisense DNA. *J Chromatogr A* 1993; 638: 293–301.
23. DeDionisio L. Capillary gel electrophoresis and the analysis of DNA phosphorothioates. *J Chromatogr A* 1993; 652: 101–8.
24. Bergot BJ, Egan W. Separation of synthetic phosphorothioate oligodeoxynucleotides from their oxygenated (phosphodiester) defect species by strong-anion-exchange-high-performance liquid chromatography. *J Chromatogr* 1992; 599: 35–42.
25. Metelev V, Agrawal S. Ion-exchange high-performance liquid chromatography analysis of oligodeoxyribonucleotide phosphorothioates. *Anal Biochem* 1992; 200: 342–6.
26. Huber CG, Oberacher H. Analysis of nucleic acids by on-line liquid chromatography-mass spectrometry. *Mass Spectrom Rev* 2001; 20: 310–43.
27. Bothner B, Chatman K, Sarkisian M, Siuzdak G. Liquid chromatography mass spectrometry of antisense oligonucleotides. *Bioorg Med Chem Lett* 1995; 5: 2863–8.
28. Tengvall U, Auriola S, Antopolsky M, Azhayev A, Biegelman L. Characterization of antisense oligonucleotide-peptide conjugates with negative ionization electrospray mass spectrometry and liquid chromatography-mass spectrometry. *J Pharm Biomed Anal* 2003; 32: 581–90.
29. Gaus HJ, Owens SR, Winniman M, Cooper S, Cummins LL. On-line HPLC electrospray mass spectrometry of phosphorothioate oligonucleotide metabolites. *Anal Chem* 1997; 69: 313–19.
30. Griffey RH, Greig MJ, Gaus HJ, et al. Characterization of oligonucleotide metabolism in vivo via liquid chromatography/electrospray tandem mass spectrometry with a quadrupole ion trap mass spectrometer. *J Mass Spectrom* 1997; 32: 305–13.
31. Graham MJ, Crooke ST, Lemonidis KM, et al. Hepatic distribution of a phosphorothioate oligodeoxynucleotide within rodents following intravenous administration. *Biochem Pharmacol* 2001; 62: 297–306.

32. Dai G, Wei X, Liu Z et al. Characterization and quantification of Bcl-2 antisense G3139 and metabolites in plasma and urine by ion-pair reversed phase HPLC coupled with electrospray ion-trap mass spectrometry. *J Chromatogr B* 2005; 825: 201–13.
33. Beverly M, Hartough K, Machemer L, Pavco P, Lockridge J. Liquid chromatography electrospray ionization mass spectrometry analysis of the ocular metabolites from a short interfering RNA duplex. *J Chromatogr B* 2006; 835: 62–70.
34. Capaldi DC. Ion-pair HPLC-UV-MS analysis of complex oligonucleotide drug products. Presented at: TIDES. Las Vegas, NV. May 17–20, 2009.
35. Tang L, Kebarle P. Dependence of ion intensity in electrospray mass spectrometry on the concentration of the analytes in the electrosprayed solution. *Anal Chem* 1993; 65: 3654–68.
36. Rentel C. Development of an LC-MS method for quality control of oligonucleotides. Presented at: TIDES. La Costa, CA. May 1–3, 2006.
37. Rentel C, Wang X, Batt M, et al. Formation of modified cytosine residues in the presence of depurinated DNA. *J Org Chem* 2005; 70: 7841–5.
38. Capaldi DC, Gaus HJ, Krotz AH, et al. Synthesis of high quality antisense drugs. Addition of acrylonitrile to phosphorothioate oligonucleotides: adduct characterization and avoidance. *Org Proc Res Dev* 2003; 7: 832–8.
39. Gaus HJ, Olsen P, Van Sooy K, et al. Trichloroacetaldehyde modified oligonucleotides. *Bioorg Med Chem Lett* 2005; 15: 4118–24.
40. Kossel A, Neumann A. Ueber das Thymin, ein Spaltungsproduct der Nucleinsäure. *Ber. Chem Ges* 1893; 26:2753–6.
41. Zoltewicz, JA, Clark DF, Sharpless TW, Grahe G. Kinetics and mechanism of the acid-catalyzed hydrolysis of some purine nucleosides. *J Am Chem Soc* 1970; 92: 1741–50.
42. Garrett ER, Mehta PJ. Solvolysis of adenine nucleosides: I. Effect of sugars and adenine substituents on acid solvolyses. *J Am Chem Soc* 1972; 94: 8532–41.
43. Garrett ER, Seydel JK, Sharpen AJ. The acid-catalyzed solvolysis of pyrimidine nucleosides. *J Org Chem* 1966; 31: 2219–27.
44. Oivanen M, Kuusela S, Lonnberg H. Kinetics and mechanism for the cleavage and isomerization of the phosphodiester bonds of RNA by Bronsted acids and bases. *Chem Rev* 1998; 98: 961–0.
45. Tamm C, Chargaff E. Physical and chemical properties of the apurinic acid of calf thymus. *J Biol Chem* 1953; 203: 689–94.
46. Bradley K, Kurata C, Rentel C, Capaldi DC. Unpublished results.
47. Halliwell B, Aruoma OI. DNA damage by oxygen-derived species FEBS. 1991; 281: 9–19.
48. Dizdaroglu M. Chemical determination of free radical-induced damage to DNA. *Free Radical Biol Med* 1991; 10: 225–42.
49. Marnett LJ. Oxyradicals and DNA damage. *Carcinogenesis* 2000; 21: 361–70.
50. Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxidants and the degenerative diseases of aging. *Proc Natl Acad Sci USA*. 1993; 90: 7915–22.
51. Ames BN, Gold LS, Willett WC. The causes and prevention of cancer. *Proc Natl Acad Sci USA* 1995; 92: 5258–65.
52. Halliwell B. Oxidants and human disease: some new concepts. *FASEB J* 1987; 1: 358–64.
53. Pogozelski WK, Tullius TD. Oxidative strand scission of nucleic acids: routes initiated by hydrogen abstraction from the sugar moiety. *Chem Rev* 1998; 98: 1089–107.
54. Aruoma OI, Halliwell B, Gajewski E, Dizdaroglu M. Damage to the bases in DNA induced by hydrogen peroxide and ferric iron chelates. *J Biol Chem* 1989; 264: 20509–12.
55. Bradley K, Kurata C, Rentel C, Capaldi DC. Unpublished results.
56. Gordillo B, Salas M, Hernandez J. The lack of influence of electronic effects on the stereochemistry of the oxidation of aryl thiophosphates. *J Chem Soc, Perkin Trans. 2*. 1999: 1281–6.
57. Gish G, Eckstein F. DNA and RNA sequence determination based on phosphorothioate chemistry. *Science* 1988; 240: 1520–2.
58. Lindahl T, Nyberg B. Rate of depurination of native deoxyribonucleic acid. *Biochemistry* 1972; 11: 3610–8.
59. Lindahl T, Nyberg B. Heat induced deamination of cytidine residues in deoxyribonucleic acid. *Biochemistry* 1974; 13: 3405–10.
60. Lindahl T. Irreversible heat inactivation of transfer ribonucleic acids. *J Biol Chem* 1967; 242: 1970–3.
61. Shapiro R. Damage to DNA caused by hydrolysis. In: Seeberg E, Kleppe K, eds. *Chromosome Damage and Repair*. New York: Plenum, 1981: 3–12.

62. Suzuki T, Ohsumi S, Makino, K. Mechanistic studies on depurination and apurinic site chain breakage in oligodeoxynucleotides. *Nucleic Acids Res* 1994; 22: 4997–5003.
63. Karran P, Lindahl T. Hypoxanthine in deoxyribonucleic acid: generation by heat-induced hydrolysis of adenine residues and release in free form by a deoxyribonucleic acid glycosylase from calf thymus. *Biochemistry* 1980; 19: 6005–11.
64. Garrett ER, Tsau J. Solvolyses of cytosine and cytidine. *J Pharm Sci* 1972; 61: 1052–61.
65. Frederico LA, Kunkel TA, Ramsay Shaw B. A sensitive genetic assay for the detection of cytosine deamination: determination of the rate constants and the activation energy. *Biochemistry* 1990; 29: 2532–7.
66. Ehrlich M, Norris KF, Wang RY-H, Kuo KC, Gehrke CW. DNA cytosine methylation and heat-induced deamination. *Biosci Rep* 1986; 6: 387–93.
67. Westheimer FH. Why nature chose phosphates. *Science* 1987; 235: 1173–8.
68. Schroeder GK, Lad C, Wyman P, Williams NH, Wolfenden R. The time required for water attack at the phosphorus atom of simple phosphodiester and of DNA. *Proc Natl Acad Sci USA* 2006; 103: 4052–5.
69. Lonneberg H. Personal communication.
70. Krishnamoorthy S, Bradley K, Capaldi DC. Unpublished results.
71. Kirby AJ, Varvoglis AG. The reactivity of phosphate esters. Monoester hydrolysis. *J Am Chem Soc* 1967; 89: 415–23.
72. Burnotte J, Verly WG. Crosslinking of methylated DNA by moderate heating at neutral pH. *Biochim Biophys Acta* 1972; 262: 449–52.
73. Goffin C, Verley WG. Interstrand DNA crosslinks due to AP (apurinic/apyrimidinic) sites. *FEBS*. 1984; 161: 140–4.
74. Dutta S, Chowdhury G, Gates KS Interstrand cross-links generated by abasic sites in duplex DNA. *J Am Chem Soc* 2007; 129: 1852–3.
75. Capaldi DC. Practical concerns in oligonucleotide stability. Presented at: Oligonucleotide-based Therapeutics. Bethesda, MD. April 19–20, 2007.
76. Rahgozar G, Capaldi DC. Unpublished observations.
77. Ullman JS, McCarthy BJ. Alkali deamination of cytosine residues in DNA. *Biochim Biophys Acta* 1973; 294: 396–404.
78. Garrett ER, Yakatan GJ. Solvolysis of 5-halonucleosides and related nucleosides. *J Pharm Sci* 1968; 57: 1478–87.
79. Garrett ER, Mehta PJ. Solvolysis of adenine nucleosides: II. Effects of sugars and adenine substituents on alkaline solvolyses. *J Am Chem Soc* 1972; 94: 8542–7.
80. Jones AS, Mian AM, Walker RT. The action of alkali on some purines and their derivatives. *J Chem Soc (C)*. 1966: 692–5.
81. Li Y, Breaker RR. Kinetics of RNA degradation by specific base catalysis of transesterification involving the 2'-hydroxyl group. *J Am Chem Soc* 1999; 121: 5364–72.
82. Luu N, Capaldi DC. Unpublished observations.
83. Rahgozar G, Capaldi DC. Unpublished observations.
84. Cadet J, Sage E, Douki T. Ultraviolet radiation-mediated damage to cellular DNA. *Mut Res* 2005; 571: 3–17.
85. Pfeifer GP, You Y-H, Besaratinia A. Mutations induced by ultraviolet light. *Mut Res* 2005; 571: 19–31.
86. Cadet J, Courdavault S, Ravanat J-L, Douki T. UVB and UVA radiation-mediated damage to isolated and cellular DNA. *Pure Appl Chem* 2005; 77: 947–61.
87. Setlow RB, Carrier WL. Pyrimidine dimers in ultraviolet-irradiated DNA's. *J Mol Biol* 1966; 17: 237–54.
88. Ravanat J-L, Douki T, Cadet J. Direct and indirect effects of UV radiation on DNA and its components. *J Photochem Photobiol B* 2001; 63: 88–102.
89. The existence of photoproducts other than CPD was first reported by Johns HE, Pearson ML, LeBlanc JC, Helleiner CW. The ultraviolet photochemistry of thymidyl-(3'→5')-thymidine. *J Mol Biol* 1964; 9: 503–24.
90. Taylor JS, Cohrs MP. DNA, light, and Dewar pyrimidinones: the structure and biological significance to TpT3. *J Am Chem Soc*, 1987; 109: 2834–5.
91. Boorstein RJ, Hilbert TP, Cunningham RP, Teebor GW. Formation and stability of repairable pyrimidine photohydrates in DNA. *Biochemistry* 1990; 29: 10455–60.
92. Jiang Y, Rabbi H, Kim M et al. UVA generates pyrimidine dimers in DNA directly. *Biophysical J* 2009; 96: 1151–8.
93. Foote CF. Definition of type I and type II photosensitized oxidation. *Photochem Photobiol* 1991; 54: 659.

94. Cadet J, Beger M, Douki T, et al. Effects of UV and visible radiation on DNA – final base damage. *Biol Chem* 1997; 378: 1275–86.
95. Sheu C, Foote CS. Photosensitized oxygenation of a 7, 8-dihydro-8-oxoguanosine derivative. Formation of dioxetane and hydroperoxide intermediates. *J Am Chem Soc* 1995; 117: 474–7.
96. Duarte V, Gasparutto D, Yamaguchi LF, et al. Oxaluric acid as the major product of singlet oxygen-mediated oxidation of 8-oxo-7,8-dihydroguanine in DNA. *J Am Chem Soc* 2000; 122: 12622–8.
97. Raoul S, Cadet J. Photosensitized reaction of 8-oxo-7,8-dihydro-2'-deoxyguanosine: identification of 1-(2-deoxy- β -d-erythro-pentofuranosyl)cyanuric acid as the major singlet oxygen oxidation product. *J Am Chem Soc* 1996; 118: 1892–8.
98. Luo W, Muller JG, Rachlin EM, Burrows CJ. Characterization of spiroimidohydantoin as a product of one-electron oxidation of 8-oxo-7,8-dihydroguanosine. *Org Lett* 2000; 2: 613–16.
99. Luu N, Capaldi DC. Unpublished observations.
100. Olsen P, Capaldi DC. Unpublished observations.

16 | Stress testing to determine liposome degradation mechanisms

Paul R. Meers and Patrick L. Ahl

INTRODUCTION

Liposomes represent one of the first “nanoscale” technologies used in drug delivery, and continue to be a source of innovative solutions because of the vast variety of lipid compositions, size, charge, and other characteristics that can be tailored to a given delivery problem. In addition, the relatively nontoxic nature of many liposomal compositions has been well established. With the recently renewed importance of delivery vehicles for biopharmaceutical therapeutics with intracellular targets such as siRNA, liposomes continue to be an attractive nontoxic formulation choice that can function well in many situations.

Truly liposomal formulations consist of a supramolecular aggregate of lipids in one or more bilayers that completely enclose or encapsulate an aqueous space (Fig. 1). These vesicular structures arise from the tendency for many lipids to organize in lamellar states referred to as smectic mesophases. This organization requires specific structural properties of the lipids and specific interactions between the lipids. In general, amphiphatic lipids with segregated hydrophobic and hydrophilic chemical groups, such as glycerolipids, are often bilayer forming. The phase behavior of lipids is highly dependent on the details of the chemical structure, and even small changes, such as the protonation of an acidic group can cause a phase change.

The liposome depicted in Figure 1 is an iconic representation that does not encompass the actual structural diversity that is possible in liposomal formulations. Liposomes can have varying bilayer thickness, bilayer lipid packing, surface charge, number of lamellae, and can vary in the overall diameter and shape. These variations can occur even within a given liposomal preparation. Nonetheless, methods have been devised (as discussed below) to control and analyze these variations, such that stable and reproducible drug delivery formulations can be developed.

Typically aqueous-soluble components are encapsulated inside the liposome for sustained and/or targeted release after administration. Alternatively, hydrophobic compounds can be associated with the lipid bilayer itself. A third alternative is a complex of liposomes with an active drug, such as the lipid complex Abelcet® or any of the abundant cationic liposomal complexes with polynucleotides that are used for plasmid or siRNA delivery.

Liposomes themselves have an active therapeutic role in the sense that they must deliver the active drug that is associated with them. Because they are macromolecular assemblies, it is the physical characteristics of the assembly that primarily determine the delivery activity. However, the detailed chemistry of the components ultimately dictates the physical properties of the whole liposome. Conversely, it is the physical and structural properties of the liposome that dictate the stability of the lipid components, as we will describe below. In this sense, the stability issues in liposomes are unique and significantly different from small molecule drugs.

In this chapter, we will offer a collection of some of the publicly available information on methods that may be used to test liposomal formulations under stress. Though not comprehensive, it is the intention to supply a useful reference to help assemble stress-testing protocols.

CHEMICAL STABILITY OF LIPOSOMES

The most common components of liposomes used in pharmaceuticals are naturally occurring phospholipids and cholesterol. Phospholipids are built on a glycerol backbone. Two hydrophobic long acyl chains are esterified to the *sn*-1 and *sn*-2 positions, and a phosphoester occupies the *sn*-3 position, as in 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine or DPPC as

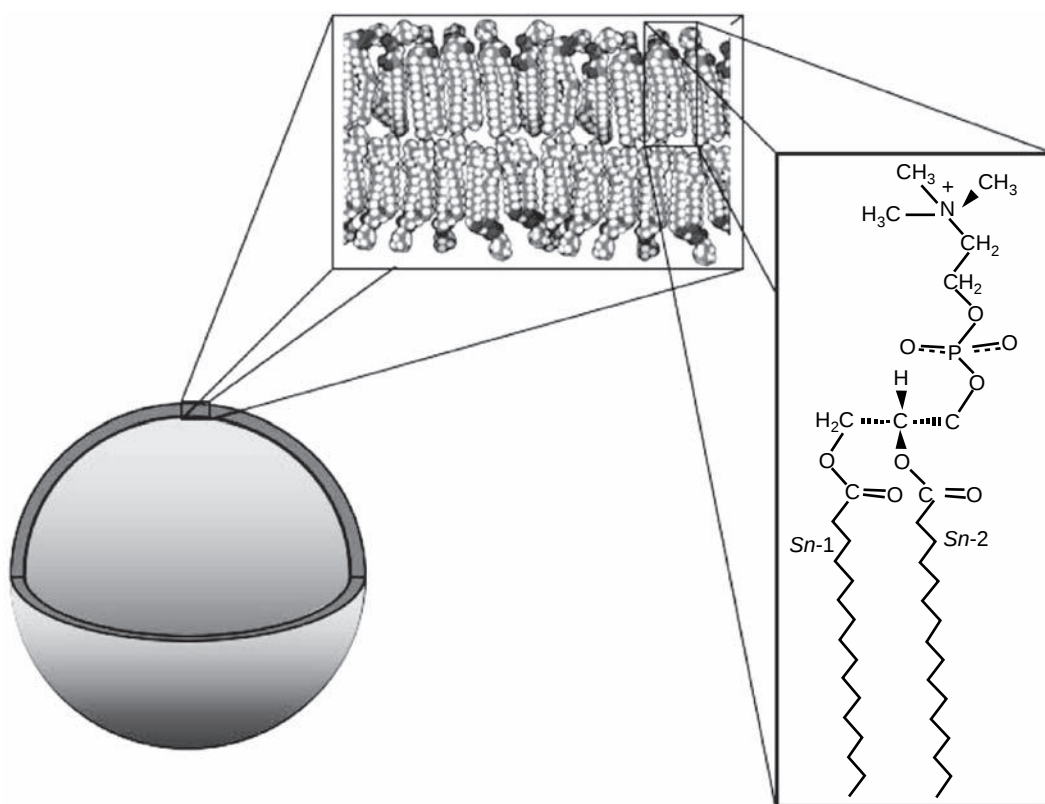


Figure 1 Schematic representation of a liposome. A liposome composed of the lipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) with a diameter of approximately 100 nm and a membrane thickness (phosphorus to phosphorus) of approximately 3.8 nm is shown. Top inset shows a bilayer with space filling models of DPPC arranged in a typical bilayer organization. Far right inset shows the chemical structure of DPPC. The stereo specific numbering (*sn*) for the glycerol backbone attachment of acyl chains is also shown. Source: Adapted from public domain figure at http://en.wikipedia.org/wiki/File:Lipid_bilayer_section.gif

shown in Figure 1. Uniquely characteristic hydrophilic head groups are also esterified to the glycerophosphate at the *sn*-3 position, choline in the case shown (Fig. 2). Phospholipid bilayers are stabilized by the enthalpic contributions from the van der Waals interactions between the long aligned acyl chains and the hydrogen bonds formed between the hydrophilic head groups and the bulk water. There is also an entropic contribution to ΔG from the sequestration of the hydrophobic acyl moieties from the aqueous medium, avoiding ordering of water molecules along exposed hydrophobic surfaces. The detailed structures of these molecules are critical to their ensemble behavior. Liposomes do not assemble from phospholipids with only one acyl chain or chains less than approximately 10 carbons. They also do not form from triacylglycerols or other lipids that do not have an adequate head group area to structurally match the hydrophobic portion. Therefore, the packing of lipids into stable nonleaky bilayers is highly sensitive to changes in chemical structure.

Cholesterol is also an important component in many liposomal formulations. Its unique structure gives it properties that are usually stabilizing to phospholipid bilayers. Cholesterol interacts with phospholipid acyl chains in such a way as to essentially buffer the gel-to-liquid crystalline phase transition that is characteristic of pure phospholipid bilayers. In this way, cholesterol can suppress instability and leakage known to occur under conditions that place phospholipid bilayers at or near this phase transition. Cholesterol also tends to decrease the

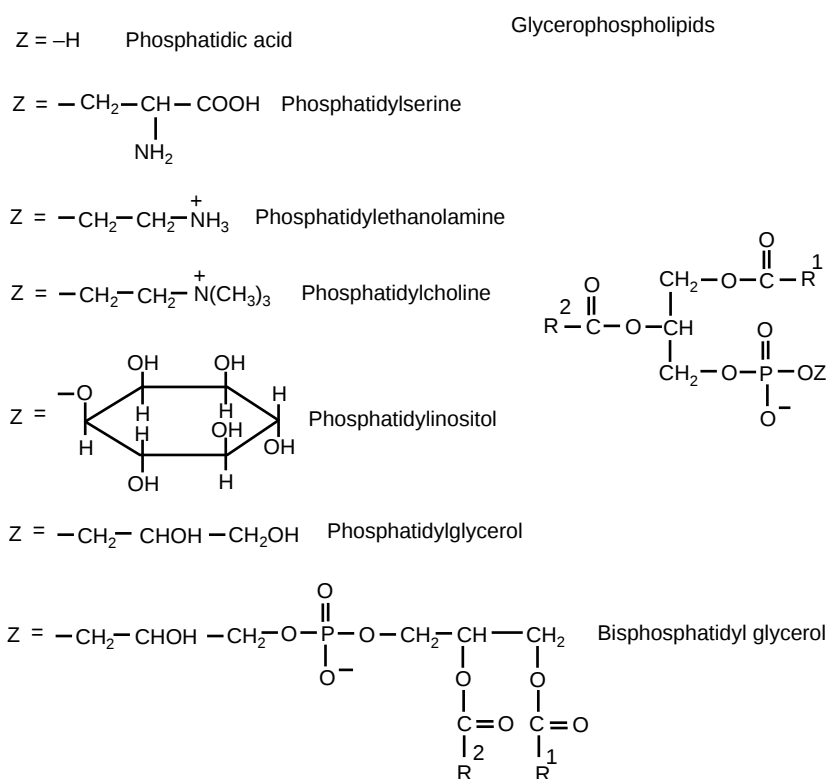


Figure 2 Chemical structures of some naturally occurring glycerophospholipids. The glycerol backbone of these molecules along with esterified acyl chains are shown in the upper right. Z represents the various polar head group esterified to the phosphoryl group at the *sn*-3 position of glycerol. *Source:* Adapted from public domain figure at <http://en.wikipedia.org/wiki/File:Glycerophospholipids.png>

"leakiness" of liposomal membranes at all temperatures. Therefore, the chemical integrity of cholesterol is also important in the overall physical stability of liposomes.

Phospholipids with polyethylene glycol moieties (PEG) became popular for liposome formulations beginning more than 20 years ago. In these lipids, a large hydrophilic 1 to 5 kDa PEG group is covalently attached, usually to the amino group of phosphatidylethanolamine. Other hydrophobic anchors have been investigated as well. When these lipids are incorporated into liposomal membranes, usually at only a small percentage of the total lipid, the hydrophilic PEG is localized on the surface of the liposome and can essentially cover all of the membrane lipids. In this way, it has been possible to dramatically change the properties of liposomes, particularly with respect to interactions with interfering agents in the biological milieu, such as serum proteins (1). However, PEG lipids present unique problems in stability testing as will be discussed below.

In the following sections, we will present a noncomprehensive discussion of some of the primary degradation processes in liposomes with a few relevant examples.

HYDROLYSIS

One of the most prominent pathways of spontaneous degradation of phospholipids is hydrolysis of the least stable bonds in the molecule, which are the ester bonds of the *sn*-1 and *sn*-2 acyl chains. Common early degradants of phospholipids, fatty acids, and lyso-phospholipids

released by hydrolysis, have important consequences for the overall physical stability of the liposome. Most commonly, the *sn*-2 chain is hydrolyzed first, leaving a fatty acid and a lyso-lipid with a *sn*-1 acyl chain.

Although appropriately matched fatty acids and lyso-phospholipids can sometimes still exist as bilayers, they are always less stable than the parent molecule, particularly once the liposome makes contact with biological fluids where fatty acids and lysolipids can exchange rapidly. Grit and Crommelin (2) found that hydrolysis of a small percentage (~10%) of phospholipid in liposomes composed of partially hydrogenated egg phosphatidylcholine (PHEPC) and egg phosphatidylglycerol (EPG) actually depressed the leak-in rate of external calcein, a fluorescent fluid phase marker. However, at higher percentages of hydrolysis or on the addition of exogenous lyso-phosphatidylcholine alone without fatty acid, the leak-in rate was increased. Ultimately, high levels of these two detergents can lead to disintegration of liposomes into micelles (3). This is highly dependent on the liposomal composition. For instance, liposomes containing PEG lipids (see below) can disintegrate with as little hydrolysis as 3.6% of the total phospholipids in the membrane (4).

The major factor in the hydrolysis of soluble esters is the solution pH, and this holds true for liposomes as well (Fig. 3). In general, acidic pH promotes the nucleophilic attack by water on the protonated ester while the nucleophilic attack by a hydroxyl ion is promoted at basic pH in an addition-elimination reaction. However, the structure and physical properties of the liposome greatly affect pH-dependent hydrolysis. Because of the bilayer structure (Fig. 1), the local pH and exposure to aqueous solvent is dependent on the physical characteristics of the bilayer. As pointed out by Grit and Crommelin (5), the local pH at the surface of the bilayer is highly dependent on the surface charge. Protons either concentrate or are depleted at the surface as a result of the charge of the lipid head groups in the bilayer.

This effect can be estimated using the Guoy-Champman equation, taking into account screening effects of other ions. An effective pH is derived from the effective surface potential

$$\Delta\text{pH} = e \psi_{\text{eff}} / 2.3kT$$

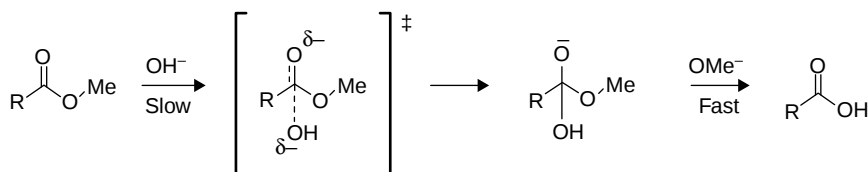
where e is the electron charge, ψ_{eff} is an effective surface potential (calculated), k is the Boltzmann constant, and T is the absolute temperature. The effective surface potential is calculated from the charge density of lipids in the bilayer, the valency, and concentration of each ion in solution, and their intrinsic binding constants for the bilayer lipids.

The general expectation from these considerations is that the bulk pH dependence for acid hydrolysis observed in neutral liposomes would shift to higher pH values for anionic liposomes and lower pH values for cationic liposomes because the surface pH would be lower for the anionic liposomes and higher for the cationic liposomes. This was in fact observed (5). For instance, the rate of hydrolysis of a neutral phospholipid was the same at pH 5 in a liposome with only neutral phospholipids as it was at pH 6 in a liposome with 40% negatively charged phospholipids (5). Somewhat more complex behavior has been seen in cationic liposomes and has been attributed to specific interactions of amino groups (6).

The ionic environment also plays a role in addition to the surface charge because of screening by the presence of other ions that localize at the surface based on their charge. This then implies that the presence of salt can help us to stabilize charged liposomes in terms of hydrolysis. Indeed, studies of varying ionic strength (5,7) showed a decreased rate of hydrolysis of charged liposomes in proportion to the neutralization of surface pH due to the reduction of surface potential by salt ions. There was no effect on neutral liposomes. In general, no matter what the surface charge or ionic strength, the hydrolysis data from Grit and Crommelin (5) scaled well with surface pH.

The local dielectric constant also plays a role in reaction rate constants. A lower dielectric constant may stabilize less polar transition states. This dielectric effect can be seen on dissociation constants of membrane-bound molecules, as even in zwitterionic lipid membranes the effective pK_a for fatty acids in the membrane is 1–2 units lower than the intrinsic pK_a (8).

Base catalyzed ester hydrolysis:



Acid catalyzed ester hydrolysis:

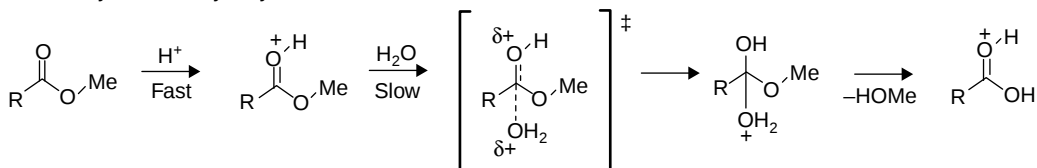


Figure 3 Hydrolysis of esters as represented by a methyl ester. Representation of acid and base catalyzed hydrolysis. In this figure, the methyl group Me is in the same bonding position as the glycerol moiety of the phospholipids would be. The R group represents the long hydrocarbon portion of the acyl chain as in Figure 2. Basic hydrolysis leads to a transition state with partial negative charges. Acidic hydrolysis leads to a transition state with partial positive charges. The localization of these bonds in the interfacial region of the membrane, that is, between the aqueous phase and the hydrocarbon interior (Fig. 1) affects the rate of hydrolysis. *Source:* Adapted from public domain figure at http://commons.wikimedia.org/wiki/File:Wikipedia_ester_hydrolysis.png

The stability of the *sn*-1 and 2 *sn*-2 esters is also sensitive to bilayer packing. Accessibility of the ester to attacking water molecules can vary considerably depending on the state of the bilayer. In many respects the lamellar organization is protective of these esters. Phospholipids in mixed micelles were found to hydrolyze much more rapidly than those in bilayers (9), presumably due to the greater accessibility of water molecules to the ester bonds in the loosely packed micelles. It was also observed that pure saturated chain phosphatidylcholine liposomes show a break in the Arrhenius plots of hydrolysis near the gel-to-liquid crystalline phase transition temperature, with higher activation energy in the gel phase (10) presumably due to tighter lipid packing. Therefore, the state of chain packing should be considered in the design of liposome testing conditions.

In light of these considerations, it is clear that the pH and ionic conditions chosen for stress testing of liposomes will depend strongly on the lipid composition being tested. In one specific example, Zhang and Pawelchak (7) reported a multifactorial study on the hydrolytic stability of zwitterionic liposomes of egg phosphatidylcholine and cholesterol using pH values of 4.0 and 4.8 with temperatures of 30°C, 40°C, or 50°C. Ionic strength and headspace oxygen were also varied, the latter to test oxidation effects. Lipid integrity was analyzed with an Astec diol bonded phase column 250 × 4.6 mm (advanced separations) and an evaporative light scattering detector (ELSD). The mobile phase consisted of chloroform, methanol, and water at a volume ratio of 65:25:4, respectively. This is a classic mobile phase and support in column chromatography of phospholipids, also commonly used in thin layer chromatography. These authors also found a critical role for ionic strength in stability of these liposomes.

In another more recent study (11) on the liposomal products Doxil® (12,13) and DaunoXome® (14,15) which contain encapsulated anthracyclines (refer to Table 1 for composition details), samples were stressed for six days at 50°C, with 1% H₂O₂, or under acidic pH (~3), or basic pH (~8.5) conditions. Different methods of analysis were used. The change in lipid composition was monitored by reverse phase HPLC on a Chromegabond PEP 5µm 250 × 4.0 mm id column at 40°C. The run time was 22 minutes with an ethanol:water gradient at a flow rate of 1.0 mL/min. Detection with ELSD was achieved with N₂ flow of 2.2 L/min,

Table 1 Some Liposomal Products that have Been Studied Under Stress Conditions

Product	Drug	Liposome Size	Lipid Composition (wt%)	Additional Solutes
Doxil®	Doxorubicin hydrochloride (encapsulated by remote loading)	Approx. 100 nm diameter	- Hydrogenated soybean phosphatidylcholine (56.2) - Cholesterol (38.3) - Polyethylene-glycol (Mr 1,900) derivatized distearoyl-phosphatidylethanolamine (5.3) α-tocopherol (0.2)	2 mg/mL ammonium sulfate (different inside and outside liposome)
DaunoXome®	Daunorubicin citrate (encapsulated)	Approx. 40–60 nm diameter	1,2-distearoyl-<i>sn</i>-glycero-3-phosphocholine (66.7) - Cholesterol (33.3)	5% dextrose
AmBisome®	Amphotericin B (intercalated into lipid bilayer)	<100 nm diameter	- Hydrogenated soybean phosphatidylcholine (60.9) - Cholesterol (14.9) 1,2-distearoyl-<i>sn</i>-glycero-3-phosphoglycerol (24) α-tocopherol (0.2)	75 mg/mL sucrose; 2.25 mg/mL Disodium succinate hexahydrate pH 5–6

nebulizer temperature of 35°C, evaporative temperature of 55°C, and a gain of 500 V. Acidic and basic conditions showed the greatest degradation of the lipids, notably the appearance of lyso-phosphatidylcholine species and degradants of cholesterol. Decomposition was most pronounced under acidic conditions in the range of pH 3. These methods are probably more typical of what is currently used in the pharmaceutical industry for these types of liposomes. However, as we have emphasized, the most informative stress conditions will depend on the type of liposomes being analyzed.

In recent years, charged aerosol detectors (CAD) have become a promising and increasingly popular method of detection for HPLC of lipids. In the CAD, the column eluent is nebulized to small droplets and the mobile phase evaporated by a nitrogen stream. The resulting particles are charged by a second stream passed through a platinum corona charger. The charged particles are detected by a sensitive electrometer (refer to chap. 9 in this book on “mass balance” for more detail). These devices have the advantage of relatively linear and proportional scaling of response to the mass of all species. This is not the case for ELSD, which has thus far been a popular choice for lipid analysis because of its sensitivity and lack of dependence on a chromophore. Recent work has shown the utility of the CAD to monitor phospholipid hydrolysis (16), detect PEG lipids (17), and phospholipids in biological samples (18).

After the relatively rapid hydrolysis of fatty esters that is initially observed, other, less common degradation pathways can occur, including the hydrolysis of the more stable phosphate ester linkages. The acid or base-catalyzed hydrolysis of the alcohol head group is most likely the next step of degradation, leading to the generation of 3-glycerophosphate. This product is then prone to form the 2,3-cyclic ester in acidic and basic conditions. The cyclic ester then opens to give an equilibrium mixture of the 2- or 3-phosphoesters as shown in Figure 4 (19).

PEG-lipids have gained importance as constituents of many liposomal formulations, including Doxil®. Typically, PEG is coupled to a phosphatidylethanolamine molecule via a carbamate linkage to the amino group of this lipid. This is a relatively stable bond compared to the *sn*-1 and *sn*-2 esters, but it can be hydrolyzed under stress conditions (20). A comparison of the succinyl, amide, and carbamate linkages showed that the succinyl linkage was the most labile (21).

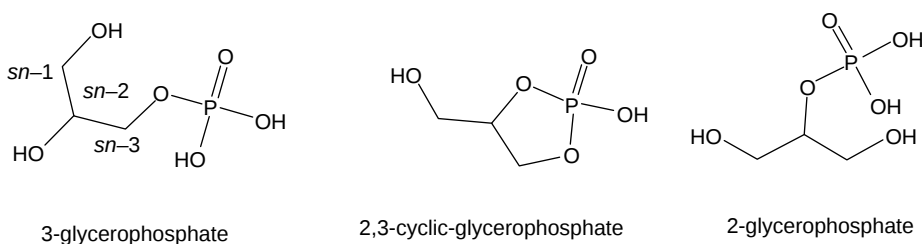


Figure 4 Cyclization of glycerophosphate from hydrolyzed phospholipids. After loss of fatty acids originally esterified at the *sn*-2 position or both the *sn*-1 and *sn*-2 positions, hydrolysis of the headgroup generates 3-phosphoglycerol. Subsequent intramolecular dehydration forms the cyclized diester, which can then hydrolyze to either the 2 or 3 phosphoglycerol.

Mass spectrometric (MS) methods are an important type of analysis for PEG-lipid conjugates. However, because of the large and polydisperse PEG groups, analytical methods for PEG lipids can sometimes be complicated. In particular, the presence of numerous species with different masses and net charges can occur in the MS analysis. In addition to the increasingly useful MS methods for synthetic polymers (22,23) some other unique methods have been devised as further aids to monitor hydrolysis. One example is the development of a Fourier transform infrared analysis to detect hydrolysis of the acyl chain esters in liposomes containing PEG phospholipids (20).

Cationic lipids represent a relatively new and less well-characterized group of liposome-forming lipids. The number and chemical variety of this class of lipids is well beyond the scope of this review to address. However, it is worth commenting that some of the cationic lipids, such as *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium (DOTAP) also contain acyl chains esterified to alcohol groups and will have similar chemical behavior to phospholipids. In fact DOTAP hydrolysis has been observed under stressed conditions (6). Some cationic lipids are actually based on phospholipids, such as varieties with spermine-linked headgroups (24) or ethylated phosphoryl groups (25).

OXIDATION

Liposomal pharmaceuticals, such as Doxil® and AmBisome® (26) (refer to Table 1 for composition details), often contain saturated acyl chain phospholipids. Thus, phospholipid peroxidation is not a significant concern in these liposome formulations. However, peroxidation of unsaturated phospholipids and cholesterol can have a major impact on the efficacy of some liposome-based therapeutics. The weakly bonded bisallylic hydrogens in polyunsaturated fatty acids (PUFA) can be removed by excited oxygen species and free radicals (27). This is recognized in the FDA draft guideline entitled "Guidelines for Liposome Drugs Products" which specifies that tests should be developed to evaluate the oxidative stability of liposomal products (see below).

As the packing of the bilayer is critically dependent on the structure of the hydrophobic chains and the subtle van der Waals interactions between them, oxidation can play a major role in altering the physical characteristics of the liposomes (28). These changes can affect numerous biophysical properties of the liposome structure including (i) the width and/or volume of the bilayer, (ii) the bilayer microfluidity, (iii) the rate of lipid lateral diffusion, and (iv) the lateral homogeneity, that is, lipid domain, structure of the membrane. These bilayer properties control critical liposome attributes of drug and water permeability of the bilayer. For example, 2,2'-azo bis (2-amidinopropane) hydrochloride (AAPH)-induced lipid peroxidation has been shown to increase the rate of water permeation (29).

The state of the liposome can also affect oxidative chemistry. Like hydrolysis, some mechanisms of oxidation are critically dependent on the physical parameters of the liposomes, such

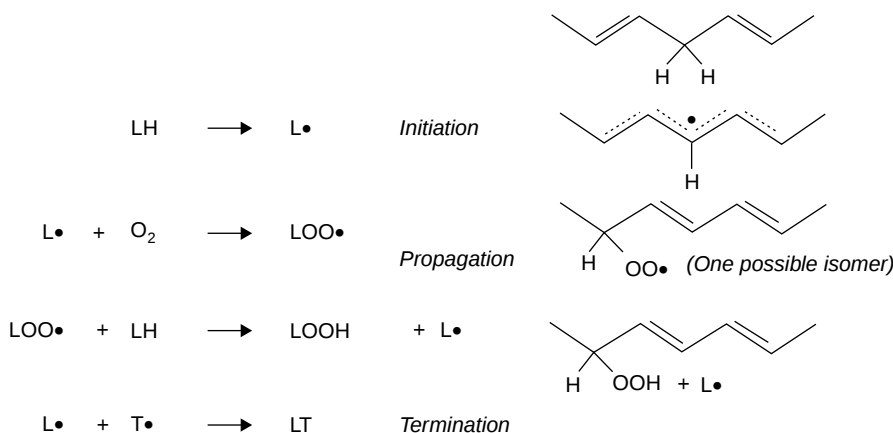


Figure 5 Peroxidation of fatty acids. Generalized initiation, propagation, and termination steps are shown on the left where L represents a lipid molecule. The right side depicts a chemical structure representative of part of the polyunsaturated fatty acyl chain in a phospholipid. Allylic hydrogens are shown and the corresponding propagation steps are shown.

as surface charge (30,31) and bilayer packing (29,32). For instance negative surface charge may attract oxidizing transition metal ions to the surface of liposomes, although the ions may also be sequestered by the same anionic lipids.

The chemistry of lipid peroxidation is complex (33). Oxidation of unsaturated phospholipids can result in a wide variety of products (34) including (i) long acyl chain products which maintain the basic phospholipid structure, (ii) lower molecular weight products formed by cleavage of unsaturated fatty acids, and (iii) adducts formed by the combination of oxidized products with other molecules containing nucleophilic groups. One fatty acid fragment product of PUFA peroxidation, malondialdehyde (MDA), is a key marker of lipid peroxidation and is potentially mutagenic and atherogenic (35). In addition to these adverse effects within the liposome bilayer, lipid free radicals generated during peroxidation could also chemically inactivate the associated therapeutic drug or protein. Conversely, the encapsulated drug can also participate in the oxidation of lipids, as exemplified by the case of doxorubicin (36).

A highly simplified description of lipid (L) peroxidation is shown in Figure 5 (for more details on unsaturated fatty acid oxidation, see chap. 3 in this book). According to the description in this figure, the extent of lipid peroxidation is controlled by the following three reactions: (i) initiation, (ii) propagation, and (iii) termination. Initiation typically involves the abstraction of a hydrogen atom from the bisallylic position of a PUFA ($LH \rightarrow L\bullet$). This extraction is done by a variety of endogenous reactive species, that is, the inducer. In normal stability study conditions the inducer would be slowly generated from O_2 via transition metal and/or photochemical-catalyzed reactions (28,33).

To better understand liposome peroxidation degradation pathways, stress stability studies should be done which significantly enhance the generation of inducer species, such as $HOO\bullet$ (refer to chap. 6 in this book). However as pointed out by Schnitzer et al. (33), the type and level of lipid peroxidation is controlled by the following: (i) the nature of the inducer, (ii) the composition and biophysical properties of the liposome, and (iii) the presence and concentration of antioxidant molecules. Designing a liposome peroxidation stress stability study, one must consider all these factors as will be discussed below. One tool that may be useful in this design is the list of reduction potentials for relevant lipids and other chemical species given by Buettner (37).

After hydrogen abstraction by an inducer (Fig. 5) the unpaired electron is delocalized over several carbons. One of several peroxy PUFA radical isomers ($LOO\bullet$) is then rapidly

formed by reaction with the dissolved molecular oxygen which tends to be relatively high in the hydrophobic portions of the liposomal membranes. Once the peroxy PUFA radical ($\text{LOO}^\bullet$) is formed, the oxidation of other lipids is propagated via the collisions of $\text{LOO}^\bullet$ with other PUFA (LH); this results in hydrogen abstraction and the concomitant formation of a hydroperoxide PUPA (LOOH) and another $\text{L}^\bullet$ radical. The initiation and propagation steps are illustrated in Figure 5 showing a segment that represents the bisallylic unsaturation in PUFA such as linoleic or arachidonic acid. Notice, one of the products contains both a hydroperoxide group and a conjugated diene. Both functional groups can be used in stress stability studies to quantify the rate and extent of liposome peroxidation (38,39). This set of reactions will result in the continuous production of lipid hydroperoxides until the propagation is terminated.

In the design of liposomal formulations certain strategies are used to minimize the tendency for oxidation to occur. For instance, several mechanisms can lead to the elimination of the $\text{L}^\bullet$ species responsible for propagation of the lipid preoxidation as indicated in Figure 5. The tendency for a liposome formulation to oxidize will reflect the balance between the factors responsible for initiation, propagation, and termination of the oxidation chain reaction. The propagation and termination aspects will derive from the composition and structure of the liposome formulation. For example, antioxidants can be included to enhance the chain termination rate in the liposome. Although controlling the peroxidation propagation and termination rates through bilayer composition is important, reducing the level of the initiation steps is also critical.

Stress studies may need to be designed to promote the oxidation in liposomes that have been designed to minimize it. Under normal conditions with low rates of initiation, months or years may be required to generate significant liposome peroxidation in some cases. Although heat-induced auto-oxidation can sometimes suffice, stress-stability studies may under some conditions require the use of a reaction chain inducer to promote lipid peroxidation in the formulation. Schnitzer et al. (33) emphasize that the peroxidation process will be different for different inducers. Thus, it seems appropriate that stress stability studies should be done with several types of inducer. The following four general approaches to generating inducer levels high enough for stress stability studies seem appropriate: (i) transition metal inducers, (ii) water-soluble inducers, (iii) membrane bilayer-associated inducers, and (iv) UV irradiation. Often these factors are used in combination. Micromolar levels of transition metals like Cu^{+2} or Fe^{+3} at 37°C are probably the most appropriate inducer for most liposomes. Cu^{+2} in particular can enhance PUFA free radical production by binding to PUFA hydroperoxides (33). For example, Sargis et al. (38) used $20\text{ }\mu\text{M}$ CuSO_4 at 37°C to compare the oxidation rates of two types of liposomes. In this case PUFA oxidation level was measured by the absorbance of the conjugated diene at 234 nm .

Iron-mediated oxidation is a common issue in lipid degradation. Ferrous and ferric ions can be leached from some kinds of glass vials, particularly brown vials for light sensitive products. Therefore, it may be relevant to address the effects of Fe^{3+} and possibly Fe^{2+} ions. Because of the multiple oxidation states of iron, there can be complex interactions with lipids and particularly with additives intended as antioxidants (31,40). For instance, ascorbate and α -tocopherol, which are antioxidant additives, can actually become pro-oxidants in some situations by reducing Fe^{3+} to Fe^{2+} (31,40). In this case, a small amount of pre-existing lipid peroxide can initiate the overall reaction (31). The Fe^{2+} ion acts as a reducing agent for peroxide to generate the stronger more lipophilic oxidant $\text{R-O}^\bullet$. This radical may generate more acyl chain radicals, which avidly combine with oxygen to form more peroxides. A good description of this process in membranes is shown in Figure 6 of Fukuzawa et al. (31).

Other water-soluble oxidation inducers include AAPH. A concentration of 20 mM AAPH at 37°C was used by M.A. Soto-Arriaza et al. (29) to induce peroxidation in egg-PC large unilamellar vesicles (LUVETS) over a time course of 40 minutes. In this case, oxidation was followed by MDA production and oxygen consumption.

An example of a lipid soluble inducer is 2,2'-azo bis (2,4-dimethylvaleronitrile) (AMVN) (33). In this case, the inducer is incorporated into the lipid bilayer, where its thermal decomposition to radicals initiates the oxidation reactions.

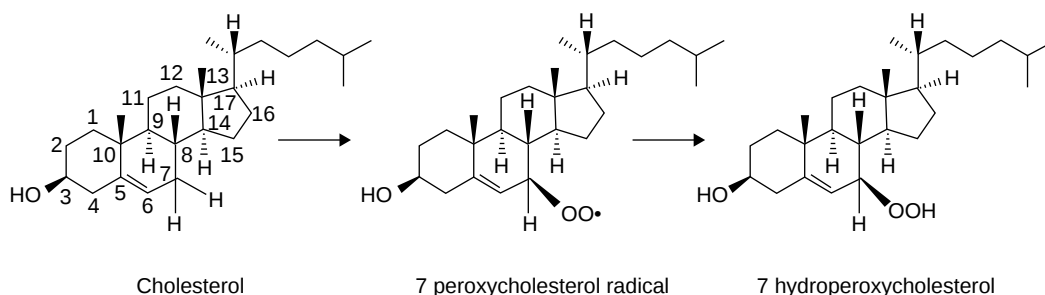


Figure 6 Peroxidation of cholesterol at position 7. Allylic hydrogen at position 7 is abstracted from cholesterol (*left*), followed by the addition of O_2 to form a 7-peroxycholesterol radical which then converts to 7-hydroperoxycholesterol abstraction of another hydrogen.

Finally, UV irradiation can be used. Polyunsaturated fatty acids, such as arachidonic acid, which may be present in lipids from natural sources, can be susceptible to oxidation as a result of exposure of the liposomal formulation to UVA irradiation (320–400 nm) (41). Most unsaturated chain phospholipids can be induced to oxidize by irradiation in the presence of membrane-associated sensitizers, such as Photofrin (42), hematoporphyrins (43) or various fluorescent molecules (44). The wavelength of irradiation in this case depends on the particular sensitizer. Photoinduced oxidation is dependent on the presence of iron and acidic pH as well (42).

Oxidation is also an important degradation reaction of cholesterol. The oxidation of cholesterol and oxidizable lipid acyl chains are often similar in mechanism and are interrelated in very complex ways when these lipids are in the same membrane (45,46). Sevanian and McLeod (47) identified several ways in which to generate oxidation of cholesterol in liposomes as well as a number of oxidation products. Oxidation was induced by atmospheric oxygen, an O_2^- generating system (ADP and $FeSO_4$), cumene hydroperoxide with hematin and $CuOOH$, or UV irradiation. Major oxidation products were analyzed using a “Diol” HPLC column and more than one eluent. These products included cholest-5-ene-3 β ol-7-one, 3,5-cholestadiene-7-one, cholestan-5 α ,6 α -epoxy-3 β ol, 5 α -6 β -triol; 3 β -hydroxycholest-5-ene-7 α -hydroperoxide, and cholestan-5 β ,6 β -epoxy-3 β -ol. They appear to derive largely from the peroxide at the seventh position (Fig. 6). This product is formed via hydrogen abstraction at the carbon to generate a three carbon allylic radical centered at this position. Subsequent oxygen addition forms the peroxide radical and then the hydroperoxide group (Fig. 6). In some situations, the degradation product resulted from direct oxy radical initiation, and in others from lipid peroxides which had formed in the unsaturated chain phospholipids used in the studies. In more recent studies, AAPH has also been used as an initiator of oxidation (45,46). A number of different methods for analysis of cholesterol oxidation products have been devised (48,49,50). Some HPLC methods that can also be used for phospholipids have also been applicable (51).

PEG lipids may also be a source or target of hydroperoxides. Although this has not had significant attention in PEG lipids, some types of PEG raw materials have been found to harbor significant amounts of hydroperoxides (52). Oxidation of PEG has been observed primarily at very high temperature when exposed to air, although some oxidation has been reported at lower temperature in aqueous solution (53). This has not been reported as a concern in pharmaceutical grade preparations of PEG containing liposomes.

BIOPHYSICAL STABILITY OF LIPOSOMES

Although the chemical decomposition of lipids in a liposome can be somewhat predictive of the physical changes, the best understanding of the biophysical stability of liposomal preparations ultimately comes from the additional measurement of these parameters directly. In fact,

in most cases the actual dependence of liposomal physical properties on changes in lipid chemistry is uncertain. There can be situations where chemical breakdown has no effect at all on a given physical property, or in the opposite direction of the expected outcome. An example, as we discussed above, is the dependence of leak on the presence of lyso-lipids and fatty acids. However, it is well demonstrated that physical parameters can change over time simply as a result of storage, for example (54). In some cases, physical parameters can change with no chemical changes whatsoever. Therefore, predictive stress testing may be important.

It is for this reason that methods for testing a characteristic set of liposomal physical parameters have been developed. The FDA has outlined some suggested parameters that could be important for determination of product stability.

In the draft FDA guidance of 2002 (55) it is stated

“The physicochemical characterization tests, which are critical to ensuring product quality of each batch of liposome drug product, should be identified. However, all the characterization tests need not be included in the specifications. Properties specific to liposome drug products that may be useful to assess include

- *morphology of the liposome, including lamellarity determination, if applicable*
- *net charge*
- *volume of entrapment in liposomal vesicles.*
- *particle size (mean and distribution profile)*
- *phase transition temperature*
- *spectroscopic data, as applicable*
- *in vitro release of the drug substance from the liposome drug product*
- *osmotic properties*
- *light scattering index”*

In general, a subset of this list is chosen, and data is collected to justify the chosen parameters as critical measures of stability that are predictive of product performance and/or toxicity. The most commonly addressed parameters are size, in vitro release of encapsulated drug, volume of entrapment, that is, captured volume, lamellarity, and surface charge. Despite the relatively simple composition of liposome formulations, the measurement of these parameters is not generally trivial. Finally, if a novel device is used to administer the liposome formulation, then device-related stress testing should be done.

LIPOSOMAL SIZE

The size of liposomes is a critical parameter with important influence on their activity and bio-distribution. Liposome size or effective size can change as a result of dissolution, aggregation or fusion of liposomes. A number of the chemical changes in liposomes can influence these processes. In addition, physical interactions with impurities or temperature changes can affect the liposomal integrity. For instance, polyvalent ions leached from packaging material can cause aggregation and fusion of charged liposomes. Therefore, the effect of stressed conditions should be monitored in terms of liposome size as assays are developed.

The size of liposomes has been estimated by a number of methods. A more extensive recent review was presented by Edwards and Baeumner (56). Many different methods, which have been used, have various advantages and disadvantages. Included among these are some methods that actually constitute a physical separation by size, such as size exclusion chromatography (57) and asymmetric field flow fractionation (58,59,60). Electron microscopic analyses by freeze fracture (61) or *cryo*-electron microscopy are often useful, particularly for morphology, on a limited number of samples, but not practical for large recurring numbers of analyses. In addition, these methods may modify the liposomes as a result of the necessary sample preparation conditions.

Light scattering methods have been widely used as a responsive, reproducible, and convenient means of assessing the stability of liposome size. In particular, photon correlation spectroscopy (or dynamic light scattering) has been used extensively because of its practicality, high sensitivity, convenience, and applicability to a wide size range, but suffers from the necessity of algorithms to distinguish and fit very small differences in the autocorrelation data that result from the measurements. In short, the movement of particles is measured from fluctuations in light scattering signal, testing the correlation between values over a range of time intervals. These functions are decaying exponentials with obviously the highest correlation at the shortest time intervals and eventually almost no correlation at the longer time intervals. The exponential decay of this function is a measure of how rapidly particles are moving and hence changing their output of scattered light to the photomultiplier tube.

On some of the most popular instruments, size distributions can be analyzed in a purely Gaussian monomodal single exponential fit or in a bimodal fit. The latter, not surprisingly, is sometimes unreliable, as pointed out by Edwards and Baeumner. In the Gaussian analysis, raw data is typically expressed as an intensity weighted average (i.e., diameter $\sum N_i I_i d_i / \sum N_i I_i$ where N_i represents the number of particles of size i , I_i their intensity and d_i their diameter). Other weighted averages, number ($\sum N_i d_i / \sum N_i$) or volume ($\sum N_i d_i^3 / \sum N_i d_i^3$), are calculated from the intensity average using Mie Theory or other modified versions. These procedures lead to some of the uncertainties in the actual meaning of each number, which have led to substantial research into improving the analyses (62,63). It is generally advisable to obtain all three averages. The correspondence between the three numbers is a measure of the polydispersity of the preparation, as in a monodisperse sample all three numbers would be the same. Light scattering will also be sensitive to the lamellarity (through refractive index), shape of particles, and contaminants such as dust particles. Some authors have presented good analyses of some of the limitations and caveats associated with photon correlation spectroscopy (64,65). Although there are unaddressed factors that affect the absolute values of diameter and distributions of diameter, this methodology can give a reasonable assessment of *changes* in size or shape as it is expected to be sensitive to these factors.

Nanoparticle tracking analysis (NTA) is new and potentially very powerful technology for measuring the particle size distributions in the 30 to 1000 nm range (66). This technology, which was commercialized in 2006 by NanoSight Ltd. (Amesbury, U.K.), uses laser light scattering microscopy to visualize and track the Brownian motion of nanoparticles such as liposomes. The particle diameter can then be determined from Brownian motion by the Stokes–Einstein equation.

An example of the importance of evaluating size changes as a result of stress conditions is the behavior of PEG lipids. There many different hydrophobically modified PEGs that have been utilized for coating liposomes. The most common are the PEG-phospholipid conjugates. Because of the very large hydrophilic portion of these conjugates relative to the hydrophobic portion, PEG lipids that are derived from conjugates with phosphatidylethanolamine can be very stable as micelles under certain conditions (67). Therefore, PEG lipids may partition out of liposomes and segregate into micelles over time without any change in the overall chemical composition of the formulation. Some other types of modified PEGs may anchor better to the bilayer, such as those with multiple hydrophobic attachments (68).

RELEASE OF ENCAPSULATED DRUG UNDER STRESS

Aqueous-soluble drugs or biopharmaceuticals are typical encapsulants in liposomes. Determination of the amount of aqueous-soluble material encapsulated inside liposomes is a key factor in product stability. Monitoring the rate of stressed release of encapsulated aqueous-soluble drugs may be one on the most sensitive and predictive methods of assessing various forms of structural or chemical degradation within liposomes. However, like the liposome size, factors other than chemical stability of the liposomal lipids themselves can affect the stability of the encapsulation of the active material.

Stress testing of release can be used at two different levels—to understand the relationship between chemical degradation and release under conditions related to storage situations or to determine the effect of degradation on release mechanisms related to the biological target. In the former situation, the release of drug can be tested at elevated temperature or upon addition of perturbants to the membrane organization, such as ions or detergents. Additionally, any methods of inducing chemical degradation, may also be applied as a stress condition for encapsulant release. The use of biological fluids related to the intended clinical target (e.g., serum) would be a first step toward a true *in vitro* release assay, which may ultimately be used as a test for manufacturing control.

In most cases of encapsulant release testing, measurements are made of the concentration of the molecules that have leaked outside the liposomes by physically separating them from those still inside. A comparison to the original encapsulated or total concentration gives an index of stressed release or leakage. Any number of methods, including many of the size analysis methods based on fractionation, can be used to attain this goal. One of the more convenient methods to obtain this liposome-free solution is centrifugal filtration of samples through a “spin-filter” device. Analysis of the filtrate gives the extraliposomal concentration of the drug or encapsulant. Prerequisites to this apparently simple analysis are related to the yield of leaked or released analyte. Because the encapsulant can bind to the separation device or even to the surface of the same liposomes from which it has leaked, careful controls must be done, including measurement of binding of analyte to empty liposomes and to the filter device itself.

Essentially the same types of experiments may be performed by asymmetric field flow fractionation, which also separates free drug/analyte from liposomes while fractionating the liposomes by size as well (58,59,60). Size exclusion column chromatography by HPLC (69) or low-pressure columns can also give information about the leakiness of stressed liposomes. However, the column methods suffer from the probability of interactions of liposomes with the support matrix (70), reducing the yield of the liposomes and in the extreme requiring cleaning or disposal of the support material. This may be less of an issue if a sizing column is used with a matrix that completely excludes liposomes, as would be sufficient for these types of experiments.

In some situations, liposomes can be removed from external solvent by ultracentrifugation. This method is normally only useful for large and multilamellar liposomes. Long run times and high force can be used to sediment smaller liposomes, but those in the range of 100 nm or less are very difficult to separate by sedimentation.

A number of probe-dependent spectroscopic techniques have been also used to measure the inside–outside distribution of a probe molecule. These would be less applicable to analyzing a real drug formulation. However, it may be informative to prepare some vesicles with probe as a matter of convenience for early studies. Probes for fluorescence (71), and NMR (72) have been developed.

An important caveat relates to the gel to liquid crystalline phase transition temperature of lipid bilayers. In bilayers of pure phospholipids, there can be a relatively sharp transition, and it is well documented that there is a substantially elevated rate of leakage of encapsulated material from these types of liposome at or near this phase transition temperature. Cycling many times through the phase transition temperature can also lead to exaggerated leakage. This may be less of an issue for membrane compositions that contain primarily unsaturated acyl chains, which have very low phase transition temperatures. Also, as we have pointed out, the essential abolition of this transition by the presence of sufficient cholesterol can obviate these issues.

ENTRAPMENT VOLUME (CAPTURED VOLUME)

The volume of entrapment per lipid or captured volume is an important indicator of not only the potential encapsulation but also the morphology of the liposome. Captured volume is typically expressed as $\mu\text{L}/\mu\text{M}$ lipid. It should not be confused with the encapsulation percentage,

which is just the encapsulated drug relative to the total drug in a formulation. Captured volume does not necessarily scale with the solute encapsulation. For instance, the hydration of multilamellar vesicles often proceeds with greater solvent penetration than solute into the interlamellar spaces (73). These measurements can also be used to study the osmotic behavior of liposome preparations by quantitative measurement of the response of average internal liposome volume to applied osmotic stress.

Several methods have been invented for this measurement (74). Again, it is desirable for stress testing to use a method in which the liposomal preparation does not have to be modified by inclusion of probes. If it is possible to use a high concentration of liposomes, then the captured volume can be assessed from a measurement of the external volume, referred to as the ViVo method. This was done previously by adding to the preformed liposomes a probe for electron spin resonance (ESR), 4-trimethylammonium TEMPO, also called CAT1. This cationic water-soluble molecule does not bind to zwitterionic lipid membranes. By measuring the apparent concentration of the CAT1 in the whole liposomal dispersion as well as the concentration of CAT1 in the supernatant after removal of liposomes, the volume occupied by the lipid bilayers and the encapsulated volume can be calculated. With known lipid concentration and density, the captured internal volume can then be determined by subtraction. It was estimated that a minimum liposome volume of about 3–4% of the total volume or a lipid concentration of about 40 mM was necessary to be able to make accurate measurements. As a cationic probe would not be compatible with anionic liposomes, other zwitterionic probes have been suggested. In principle, any aqueous soluble, membrane impermeant probe that does not interact with the liposomal membrane can be used for these measurements, including fluorescent, chromophoric or radioactive probes.

Other similar methods have been based on the capture of various solutes (75,76). If it can be demonstrated that the solute distribution actually reflects the encapsulated volume, then it may be a simpler experiment to measure the concentration of entrapped material using essentially the same methods as outlined in the section above on encapsulated solute measurements.

LAMELLARITY

Lamellarity is the number of lamellae or concentric bilayers composing the average liposome in a preparation. This is obviously important for the biological activity of the liposome as the rate of drug release or deformability of the liposome would be influenced by this parameter. Most measurements of lamellarity are approximations based primarily on the percentage or fraction (F_{out}) of lipid exposed on the outer surface of the liposomes. The actual number of lamellae would depend on their spacing and shape within the interior of the liposomes.

The average number of lamellae can be estimated in a crude way from the captured volume and mean diameter. Once these parameters are known, an estimate of lamellarity can be made based on the assumption that each lamella is equal in total volume and area, giving the average number of lamellae = $1/(2 \times F_{\text{out}})$, where F_{out} is again the average fraction of total lipid on the external monolayer of the liposomes.

In smaller liposomes, the inner bilayers will have smaller volumes and areas as a result of the smaller radius of the enclosed inner bilayer. Therefore, lamellarity may be underestimated by this formula. However, a crude index of lamellarity may be obtained by estimating the fraction of outer facing lipid F_{out} from knowledge of the captured volume (V_c) and the average size of the liposomes in terms of radius (r) and using the equation

$$F_{\text{out}} = 3V_c/a_0r,$$

where a_0 is the average area occupied per lipid molecule in the liposome bilayer. The intensity weighted value for r from photon correlation spectroscopy is the most straightforward value to use mathematically because the intensity weighting appears to scale appropriately with the phospholipid area to be used in the equation. An improved, but still uncertain, recalculated

estimate can be made by assuming that bilayers internal to the outermost bilayer are spaced inwardly by typical bilayer repeat distances obtained from X-ray diffraction.

A number of methods have been devised to estimate lamellarity based on the measurement of the fraction of surface oriented lipids in the liposomal formulation. One of the most common methods to directly measure lamellarity uses the ^{31}P NMR signal of the phospholipids to assess their organization (77). In large unilamellar vesicles, the ^{31}P NMR signal is rather broad. Addition of the paramagnetic cation Mn^{2+} and binding to the phosphate group of the phospholipids leads to drastic broadening of the signal from those lipids to which the Mn^{2+} ions are bound due to paramagnetic enhancement of the T_2 relaxation time of the ^{31}P nuclei. Because Mn^{2+} is impermeable to most membranes, only the outer facing phosphate phosphorus nuclei are affected. Disappearance of signal intensity due to broadening should be proportional to the fraction of outer phospholipids in the liposomes. The ratio of the signal intensity after Mn^{2+} to the signal intensity before Mn^{2+} gives a measure of the percentage of outer facing lipid phosphate groups, which is related to the number of lamellae in a relatively simple way as discussed above.

Unfortunately, this method is not applicable to all liposomes and is dependent on the integrity of the liposomal membrane. In the presence of negatively charged lipids, Mn^{2+} can induce aggregation, leakage, and fusion, invalidating the measurements. Furthermore, the shape of liposomes may affect the accuracy of the results (78).

For the purposes of stress testing, it would be much preferred to utilize unmodified liposomes representative of the product being developed. However, in cases where the use of probes is necessary, justification of the methods may be possible. If membrane probes are used, lamellarity can be estimated in some very sensitive ways. In one of these methods, small amounts (1 mol% or less) of lipids labeled in the head group with 7-nitro-2,1,3-benzoxadiazol-4-yl-lipid (NBD-lipid) are added to the liposome formulation. Assuming an initially random distribution, the percentage of NBD present on the outer monolayer of the liposome can be determined by their reduction by the impermeable reducing agent, sodium dithionite (79,80). In a similar method, exposed aminolipids can be reacted with the impermeant labeling agent, trinitrobenzenesulfonate, to generate a fluorescent product on the membrane surface.

SURFACE CHARGE

The surface charge of liposomes can be a critical factor in the biological activity of a liposome. This is an especially important factor in terms of binding and uptake of macrophages, penetration of charged matrices, such as biofilms and the activity of cationic complexes of plasmid DNA or siRNA (81). Therefore, the stability of this characteristic is important to investigate in many cases. A number of factors can change the effective surface charge of a liposomal formulation. For instance, a number of cationic and anionic liposomes are stabilized by inclusion of PEG lipids, which mask the surface charge of the liposomes (82). If PEG is removed from the liposomes by diffusion or hydrolysis, the zeta potential will change dramatically.

One of the most common and convenient methods to measure surface charge is by the determination of the electrophoretic mobility of the liposomes. For a given size of liposome, the hydrodynamic mobility in an electric field is dependent on the surface charge or zeta potential of the liposomes. The zeta potential is actually different from surface charge per se as it is the potential at the slip plane for the movement of the liposomes through water, which is some distance from the actual surface of the liposome. The potential at this point is ideally related to the actual surface potential by

$$\psi_x = \psi_0 \exp(-\kappa x)$$

where ψ_x is the potential at the slip plane, ψ_0 is the potential at the surface of the liposome, x is the distance of the slip plane from the surface of the liposome, and κ is a constant referred to as the Debye length. The actual relationship between the measurements and actual surface

potential can be considerably more complex (83), and it should be kept in mind that these measurements should serve primarily as a comparative index. As we had discussed above, the effective surface potential is influenced by the ionic character of the medium in which the experiment is performed. Therefore, in designing tests of the stability of the surface charge, it is important to maintain identical conditions in all the experiments. If stress experiments involve pH changes it may be necessary to adjust the pH to a standard condition for zeta potential measurements. It should also be pointed out that changes in liposome size distribution may have some effect on the measured zeta potential, even though the measurement is supposed to normalize for this effect.

DEVICES AND MECHANICAL STRESS

The effect of devices used to deliver liposomal drug formulations is an important aspect of stability testing. Several devices may be relevant for administration of a liposomal drug, such as syringes, pumps, etc. Some devices can have large effects on the integrity of liposomal membranes, leading to leakage, size change, and a number of other issues. Therefore, device evaluation can form an important part of liposomal stress testing.

As an example, nebulizers are commonly used to deliver inhaled drugs. To be inhaled efficiently and to reach the deep lung, nebulized solutions for inhalation must have an effective aerodynamic diameter of approximately 1–5 μm . Droplets of this small size are produced by generating strong shear forces in the solution to be administered. In jet nebulizers, a turbulent flow generates the shear, while in vibrating mesh nebulizers, the rapid mechanical vibration of specifically designed meshes generate the droplets.

Nebulizers can cause significant loss of encapsulated materials from the interior of liposomes as a result of the shear forces generated. Therefore, formulations must be tested under stressed conditions to understand the effect of a given nebulizer. Ultimately, specifications for the nebulizer-induced release of drug must be developed. The most telling measurements for nebulizer-treated samples are liposome size and retention of encapsulated materials.

It is well documented that nebulization usually causes substantial leakage of small aqueous-soluble molecules from liposomes. This has been modeled in a set of studies using carboxyfluorescein as a marker for release. It was found that over the time scale of several hours of nebulization as much as 10–60% of the encapsulated dye can be released, depending on the lipid composition (84). It was also shown that liposomes of smaller size (0.2 μm) were less susceptible to leakage during nebulization than larger sizes (85). Factors such as hypotonic solution, high nebulizer air pressure (for a jet-type nebulizer) and low pH promoted leakage during nebulization of the formulations tested (86). In general it is also found that the relatively newer ultrasonic vibrating mesh nebulizers cause less liposomal leakage than the older jet nebulizers (87). Obviously, these factors will depend on the specifics of the liposome composition, but give some guidelines as to what factors will stress the formulations of interest.

Another factor that may be of importance with nebulized products is the distribution of sizes and retention of contents of liposomes across the droplet size distribution produced by the nebulizer (88). Because the actual delivery of nebulized droplets into the lung has a specific-size dependence centered around 3 μm , the distribution of liposomes and their condition throughout the droplet size range is crucial to dosing. Therefore, testing the effect of stressors on the liposome distribution and characteristics as a function of the nebulized droplet sizes is of great importance. This is done by nebulizing the liposomal formulation and separating out the nebulized droplet sizes using a cascade impactor. The four most important measurements for liposomal formulations carrying encapsulated aqueous-soluble drugs would be total drug, total lipid, percentage of drug inside the liposomes and liposome size. Although each formulation is unique, some stress factors to evaluate could be long nebulization time, effect of high or low liposome concentrations or some of the same conditions as in the chemical stress tests such as the effect of low pH treatment on lability to shear stress of the nebulizer.

Table 2 Some Relevant Stress Tests for Chemical Degradation in Liposome Formulations

Test Parameter	Stress Conditions	Analytical Methods	References
Fatty ester hydrolysis	• Low (~3–4) or high pH (~8.5) • High temperature (e.g., 50°C)	• HPLC analysis of lipids with ELSD^c or CAD^d detection	(5,7,11,16,17,18)
Unsaturated lipid peroxidation	Inducers – • Heat and endogenous O₂ • H₂O₂ • Transition metals (Fe, Cu ions) • AAPH^a • Cumene hydroperoxide/hematin/CuOOH • Hematoporphyrins • Lipid-soluble inducers, e.g., AMVN^b • UV or gamma irradiation	• Detection of MDA^e • Observation of conjugated diene at 234 nm • HPLC analysis of lipid degradants with ELSD or CAD detection • MS • HPLC-MS	(7,11,27–35,37–54)

^aWater soluble inducer, 2,2'-azobis (2-amidinopropane) hydrochloride.^bLipid soluble inducer, 2,2'-azobis (2,4-dimethylvaleronitrile).^cEvaporative light scattering detector.^dCharged aerosol detector.^eMalondialdehyde.**Table 3** Some Relevant Stress Tests for Physical Properties of Liposome Formulations

Test Parameter	Stress Conditions	Analytical Methods	References
Captured volume	• Conditions that can induce chemical breakdown (see above) • Conditions that change liposome physical parameters and/or aggregation state, e.g., temperature, ionic composition (e.g., aggregation/fusion of anionic liposomes by polyvalent cations), mechanical stress (such as nebulization or extrusion)	• Vivo ESR with CAT1 • Encapsulation of water soluble probe molecule	(74,75,76)
Drug encapsulation	Same as above	• Field flow fractionation • Filtration • Size exclusion chromatography • Sedimentation • Fluorescent probes • NMR probes	(59–61,69–72)
Liposome size	Same as above	• PCS^a • Size exclusion chromatography • Field flow fractionation • Freeze fracture electron microscopy • Cryo-electron microscopy	(57–66)
Lamellarity	Same as above	• ³¹P NMR • PCS^a w/captured volume • Fluorescent probes • Chemical surface labeling	(77–80)
Surface charge	Same as above	• Electrophoretic mobility	(81–83)

^aPCS: Photon correlation spectroscopy.

SUMMARY

Stress testing of liposomes is clearly a broadly based undertaking that can involve both chemical and physical measurements. Because of the vast variety of liposomes, the particular approach to characterization of degradation pathways will depend on the particular liposomes. This review is a noncomprehensive collection of some possible methods for stress testing from publicly available information. Many product-related stress testing protocols are never published. A selective list is presented in Tables 2 and 3 showing a few recommended important characteristics that may be tested under stress.

Although no single protocol can satisfy every situation, the key parameters discussed above can be used to assemble a selected series of tests that sufficiently predicts the pertinent breakdown pathways. The method and knowledge base in this relatively new field continues to develop and can be expected to significantly improve over the coming years as more drug delivery issues are addressed by products utilizing liposomes.

REFERENCES

1. Woodle MC, Lasic DD. Sterically stabilized liposomes. *Biochim Biophys Acta* 1992; 1113: 171–99.
2. Grit M, Crommelin DJA. The effect of aging on the physical stability of liposome dispersions. *Chem Phys Lipids* 1992; 62: 113–22.
3. Zuidam N, Gouw HK, Barenholz Y, et al. Physical (in) stability of liposomes upon chemical hydrolysis: the role of lysophospholipids and fatty acids. *Biochim Biophys Acta* 1995; 1240: 101–10.
4. Ickstein LM, Sandström MC, Mayer LD et al. Effects of phospholipid hydrolysis on the aggregate structure in DPPC/DSPE-PEG2000 liposome preparations after gel to liquid crystalline phase transition. *Biochim Biophys Acta* 2006; 1758: 171–80.
5. Grit M, Crommelin DJA. The effect of surface charge on the hydrolysis kinetics of partially hydrogenated egg phosphatidylcholine and egg phosphatidylglycerol in aqueous liposome dispersions. *Biochim Biophys Acta* 1993; 1167: 49–55.
6. Vernooij EA, Kettenes-van den Bosch JJ, Underberg WJ, et al. Chemical hydrolysis of DOTAP and DOPE in a liposomal environment. *J Control Release* 2002; 79: 299–303.
7. Zhang JA, Pawelchak J. Effect of pH, ionic strength and oxygen burden on the chemical stability of EPC/cholesterol liposomes under accelerated conditions: Part 1. Lipid hydrolysis. *Eur J Pharm Biopharm* 2000; 50: 357–64.
8. Ptak M, Egret-Charlier M, Sanson A, et al. A NMR study of the ionization of fatty acids, fatty amines and *N*-acylamino acids incorporated in phosphatidylcholine vesicles. *Biochim Biophys Acta* 1980; 600: 387–97.
9. Kensil CR, Dennis EA. Alkaline hydrolysis of phospholipids in model membranes and the dependence on their state of aggregation. *Biochemistry* 1981; 20: 6079–85.
10. Zuidam NJ, Crommelin DJ. Chemical hydrolysis of phospholipids. *J Pharm Sci* 1995; 84: 1109–13.
11. Revelle L, Lipe T, Stoub W. Monitoring lipid degradation in liposomal drug products (LDPs) using HPLC with evaporative light scattering detection (ELS). From the American Association of Pharmaceutical Scientists Annual Meeting (2004). www.aapsj.org/abstracts/AM_2004/AAPS2004-001686.PDF
12. Gabizon A, Catane R, Uziely B, et al. Prolonged circulation time and enhanced accumulation in malignant exudates of doxorubicin encapsulated in polyethylene-glycol coated liposomes. *Canc Res* 1994; 54: 987–92.
13. Gabizon A, Goren D, Cohen R, et al. Development of liposomal anthracyclines: from basics to clinical applications. *J Controlled Release* 1998; 53: 275–9.
14. McTiernan A, Whelan J, Leahy M, et al. A phase II nonrandomised open-label study of liposomal daunorubicin (DaunoXome) in advanced soft tissue sarcoma. *Sarcoma* 2006; 2006(1): 1–5.
15. Petre CE, Dittmer DP. Liposomal daunorubicin as treatment for Kaposi's sarcoma. *Int J Nanomed* 2007; 2: 277–88.
16. Nair LM, Werling JO. Aerosol based detectors for the investigation of phospholipid hydrolysis in a pharmaceutical suspension formulation. *J Pharm Biomed Anal* 2009; 49: 95–9.
17. Díaz-López R, Libong D, Tsapis N, et al. Quantification of pegylated phospholipids decorating polymeric microcapsules of perfluorooctyl bromide by reverse phase HPLC with a charged aerosol detector. *J Pharm Biomed Anal* 2008; 48: 702–7.
18. Ramos RG, Libong D, Rakotomanga M, et al. Comparison between charged aerosol detection and light scattering detection for the analysis of Leishmania membrane phospholipids. *J Chromatogr A* 2008; 1209: 88–94.

19. Evstigneeva RP. Chemical stability, In: The Phospholipids Handbook, G. Cevc ed. New York, NY: CRC Press, Taylor and Francis, 1993: 323–34.
20. Vernooij EAAM, Kettenes-van den Bosch, JJ, Crommelin DJA. Fourier transform infrared spectroscopic determination of the hydrolysis of poly(ethylene glycol)-phosphatidylethanolamine-containing liposomes. *Langmuir* 2002; 18: 3466–70.
21. Parr MJ, Ansell SM, Choi LS, et al. Factors influencing the retention and chemical stability of poly(ethylene glycol)-lipid conjugates incorporated into large unilamellar vesicles. *Biochim Biophys Acta* 1994; 1195: 21–30.
22. Peacock PM, McEwen CN. Mass spectrometry of synthetic polymers. *Anal Chem* 2006; 78: 3957–64.
23. Trimpin S, Inutan ED, Herath TN, et al. Matrix-assisted laser desorption/ionization mass spectrometry method for selectively producing either singly or multiply charged molecular ions. *Anal Chem* 2010; 82: 11–15.
24. Behr JP, Demeneix B, Loeffler JP, et al. Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA. *Proc Natl Acad Sci U S A* 1989; 86: 6982–6.
25. MacDonald RC, Rakhmanova VA, Choi KL, et al. *O*-ethylphosphatidylcholine: A metabolizable cationic phospholipid which is a serum-compatible DNA transfection agent. *J Pharm Sci* 1999; 88: 896–904.
26. Adler-Moore J, Proffitt RT. AmBisome liposomal formulation, structure, mechanism of action and pre-clinical experience. *J Antimicrob Chemother* 2002; 49(Suppl. 1): 21–30.
27. Paillous N, Fery-Forgues S. Interest of photochemical methods for induction of lipid peroxidation. *Biochimie* 1994; 76: 355–68.
28. Wratten ML, van Ginkel G, van't Veld AA, et al. Structural and dynamic effects of oxidatively modified phospholipids in unsaturated lipid membranes. *Biochemistry* 1992; 31: 10901–7.
29. Soto-Arriaza M., Sotomayor CP, Lissi EA. Relationship between lipid peroxidation and rigidity in L- α -phosphatidylcholine-DPPC vesicles. *J Colloid Interface Sci* 2008; 323: 70–4.
30. Gal S, Pinchuk I, Lichtenberg D. Peroxidation of liposomal palmitoyl linoleoylphosphocholine (PLPC), effects of surface charge on the oxidizability and potency of antioxidants. *Chem Phys Lipids* 2003; 126: 95–110.
31. Fukuzawa K, Seko T, Minami K, et al. Dynamics of iron-ascorbate-induced lipid peroxidation in charged and uncharged phospholipid vesicles. *Lipids* 1993; 28: 497–503.
32. Suwa K, Kimura T, Schaap AP. Reaction of singlet oxygen with cholesterol in liposomal membranes. Effect of membrane fluidity on the photooxidation of cholesterol. *Photochem Photobiol* 1978; 28: 469–72.
33. Schnitzer E, Pinchuk I, Lichtenberg D. Peroxidation of liposomal lipids. *Eur Biophys J* 2007; 36: 499–515.
34. Domingues MRM, Reis A, Domingues P. Mass spectrometry analysis of oxidized phospholipids. *Chem Phys Lipids* 2008; 156: 1–12.
35. Rio DD, Steward AJ, Pellegrini N. A review of recent studies on malondialdehyde as a toxic molecule and biological marker of oxidative stress. *Nutr Metab Cardiovasc Dis* 2005; 15: 316–28.
36. Winterbourn CC, Gutteridge JM, Halliwell B. Doxorubicin-dependent lipid peroxidation at low partial pressures of O₂. *J Free Rad Biol Med* 1985; 1: 43–9.
37. Buettner GR. The pecking order of free radicals and antioxidants: lipid peroxidation, α -tocopherol and ascorbate. *Arch Biochem Biophys* 1993; 300: 535–43.
38. Sargis RM, Subbaiah PV. Trans unsaturated fatty acids are less oxidizable than cis unsaturated fatty acids and protect endogenous lipids from oxidation in lipoproteins and lipid bilayers. *Biochemistry* 2003; 42: 11533–43.
39. Fukuzawa K, Fujisaki A, Akai K, et al. Measurement of phosphatidylcholine hydroperoxides in solution and intact membranes by the ferric-xylenol orange assay. *Analyt Biochem* 2006; 359: 18–25.
40. Buettner GR, Jurkiewicz BA. Catalytic metals, ascorbate and free radicals: combinations to avoid. *Rad Res* 1996; 145: 532–41.
41. Gruber F, Oskolkova O, Leitner A, et al. Photooxidation generates biologically active phospholipids that induce heme oxygenase-1 in skin cells. *J Biol Chem* 2007; 282: 16934–41.
42. Schafer FQ, Buettner GR. Acidic pH amplifies iron-mediated lipid peroxidation in cells. *Free Rad Biol Med* 2000; 28: 1175–80.
43. Goyal GC, Blum A, Grossweiner LI. Photosensitization of liposomal membranes by hematoporphyrin derivative. *Canc Res* 1983; 43: 5826–30.
44. Ayuyan AG, Cohen FS. Lipid peroxides promote large rafts: effects of excitation of probes in fluorescence microscopy and electrochemical reactions during vesicle formation. *Biophys J* 2006; 91: 2172–83.

45. Tirosch O, Kohen R, Katzhendler J, et al. Oxidative stress effect on the integrity of lipid bilayers is modulated by cholesterol level of bilayers. *Chem Phys Lipids* 1997; 87: 17–22.
46. Schnitzer E, Pinchuk I, Bor A, et al. Oxidation of liposomal cholesterol and its effect on phospholipid peroxidation. *Chem Phys Lipids* 2007; 146: 43–53.
47. Sevainian A, McLeod LL. Cholesterol autoxidation in phospholipid membrane bilayers. *Lipids* 1987; 22: 627–36.
48. Sevanian A, Seraglia R, Traldi P, et al. Analysis of plasma cholesterol oxidation products using gas- and high-performance liquid chromatography/mass spectrometry. *Free Radic Biol Med* 1994; 17: 397–409.
49. Razzazi-Fazeli E, Kleinen S, Luf W. Determination of cholesterol oxides in processed food using high-performance liquid chromatography-mass spectrometry with atmospheric pressure chemical ionisation. *J Chromatogr A* 2000; 896: 321–34.
50. Korytowski W, Bachowski GJ, Girotti AW. Chromatographic separation and electrochemical determination of cholesterol hydroperoxides generated by photodynamic action. *Analyt Biochem* 1991; 197: 149–56.
51. Lang JK. Quantitative determination of cholesterol in liposome drug products and raw materials by high-performance liquid chromatography. *J Chromatogr* 1990; 507: 157–63.
52. Wasylaschuk WR, Harmon PA, Wagner G, et al. Evaluation of hydroperoxides in common pharmaceutical excipients. *J Pharm Sci* 2007; 96: 106–16.
53. Wang JQ, He LN, Miao CX, et al. The free-radical chemistry of polyethylene glycol: organic reactions in compressed carbon dioxide. *ChemSusChem* 2009; 2: 755–60.
54. duPlessis J, Ramachandran C, Weiner N, et al. The influence of lipid composition and lamellarity of liposomes on the physical stability of liposomes upon storage. *Int J Pharm* 1996; 127: 273–8.
55. Guidance for Industry, Liposome Drug Products. (Draft) Center for Drug Evaluation and Research. Food and Drug Administration. 2002, J:\GUIDANC\2191dft.doc
56. Edwards KA, Baemmer AJ. Analysis of liposomes. *Talanta* 2005; 68: 1432–41.
57. Lesieur S, Grabielle-Madelmont C, Paternostre MT, et al. Size analysis and stability study of lipid vesicles by high-performance gel exclusion chromatography, turbidity, and dynamic light scattering. *Anal Biochem* 1991; 192: 334–43.
58. Arifin DR, Palmer AF. Determination of size distribution and encapsulation efficiency of liposome-encapsulated hemoglobin blood substitutes using asymmetric flow field-flow fractionation coupled with multi-angle static light scattering. *Biotechnol Prog* 2003; 19: 1798–811.
59. Hupfeld S, Ausbacher D, Brandl M. Asymmetric flow field-flow fractionation of liposomes: optimization of fractionation variables. *J Sep Sci* 2009; 32: 1465–70.
60. Hupfeld S, Ausbacher D, Brandl M. Asymmetric flow field-flow fractionation of liposomes: 2. Concentration detection and adsorptive loss phenomena. *J Sep Sci* 2009; 32: 3555–61.
61. Hallett FR, Nickel B, Samuels C, et al. Determination of vesicle size distributions by freeze-fracture electron microscopy. *J Electron Microscop Tech* 1991; 17: 459–66.
62. Hanus LH, Ploehn HJ. Conversion of intensity-averaged photon correlation spectroscopy measurements to number-averaged particle size distributions: 1. Theoretical development. *Langmuir* 1999; 15: 3091–100.
63. Taylor TW, Srivner SM, Sorensen CM, et al. Determination of the relative number distribution of particle sizes using photon correlation spectroscopy. *Appl Opt* 1985; 24: 3713–17.
64. Fillela M, Zhang J, Newman ME, et al. Analytical applications of photon correlation spectroscopy for size distribution measurements of natural colloidal suspensions: capabilities and limitations. *Colloids Surf A* 1997; 120: 27–46.
65. Hupfeld S, Holsaeter AM, Skar M, et al. Liposome size analysis by dynamic/static light scattering upon size exclusion-/field flow-fractionation. *J Nanosci Nanotechnol* 2006; 6: 3025–31.
66. Filipe V, Hawe A, Jiskoot W. Critical evaluation of nanoparticle tracking analysis (NTA) by NanoSight for the measurement of nanoparticles and protein aggregates. *Pharm Res* 2010; 27: 796–810.
67. Edwards K, Johnsson M. Liposomes, disks, and spherical micelles: aggregate structure in mixtures of gel phase phosphatidylcholines and poly(ethylene glycol)-phospholipids. *Biophys J* 2003; 85: 3839–47.
68. Auguste DT, Prud'homme RK, Ahl PL, et al. Association of hydrophobically-modified poly(ethylene glycol) with fusogenic liposomes. *Biochim Biophys Acta* 2003; 1616: 184–95.
69. Andrieux K, Lesieur S, Ollivon M, et al. Methodology for vesicle permeability study by high-performance gel exclusion chromatography. *J Chromatogr B Biomed Sci Appl* 1998; 706: 141–7.
70. Ruysschaert T, Marque A, Duteyrat JL, et al. Liposome retention in size exclusion chromatography. *BMC Biotechnol* 2005; 10: 5–11.

71. Ellens H, Bentz J, Szoka FC. pH-induced destabilization of phosphatidylethanolamine-containing liposomes: role of bilayer contact. *Biochemistry* 1984; 23: 1532–8.
72. Zhang XM, Patel AB, de Graaf RA, et al. Determination of liposomal encapsulation efficiency using proton NMR spectroscopy. *Chem Phys Lipids* 2004; 127: 113–20.
73. Gruner SM, Lenk RP, Janoff AS, et al. Novel multilayered lipid vesicles: comparison of physical characteristics of multilamellar liposomes and stable plurilamellar vesicles. *Biochemistry* 1985; 24: 2833–42.
74. Perkins WR, Minchey SR, Ahl PL, et al. The determination of liposome captured volume. *Chem Phys Lipids* 1993; 64: 197–217.
75. Ahl PL, Chen L, Perkins WR, et al. Interdigitation-fusion: a new method for producing lipid vesicles of high internal volume. *Biochim Biophys Acta* 1994; 1195: 237–44.
76. Gruber HJ, Wilmsen HU, Schurga A, et al. Measurement of intravesicular volumes by salt entrapment. *Biochim Biophys Acta* 1995; 1240: 266–76.
77. Barsukov LI, Victorov AV, Vasilenko LA et al. Investigation of the inside-outside distribution, intermembrane exchange and transbilayer movement of phospholipids in sonicated vesicles by shift reagent NMR. *Biochim Biophys Acta* 1980; 598: 153–68.
78. Fröhlich M, Brecht V, Peschka-Süss R. Parameters influencing the determination of liposome lamellarity by ³¹P-NMR. *Chem Phys Lipids* 2001; 109: 103–12.
79. McIntyre JC, Sleight RG. Fluorescence assay for phospholipid membrane asymmetry. *Biochemistry* 1991; 30: 11819–27.
80. Gruber HJ, Schindler H. External surface and lamellarity of lipid vesicles: a practice-oriented set of assay methods. *Biochim Biophys Acta* 1994; 1189: 212–24.
81. Perrie Y, Gregoriadis G. Liposome-entrapped plasmid DNA: characterisation studies. *Biochim Biophys Acta* 2000; 1475: 125–32.
82. Woodle MC, Collins LR, Sponsler E, et al. Sterically stabilized liposomes. Reduction in electrophoretic mobility but not electrostatic surface potential. *Biophys J* 1992; 61: 902–10.
83. Egorova EM. The validity of the Smoluchowski equation in electrophoretic studies of lipid membranes. *Electrophoresis* 1994; 15: 1125–31.
84. Niven RW, Schreier H. Nebulization of liposomes: I. Effects of lipid composition. *Pharm Res* 1990; 7: 1127–33.
85. Niven RW, Speer M, Schreier H. Nebulization of liposomes: II. The effects of size and modeling of solute release profiles. *Pharm Res* 1991; 8: 217–21.
86. Niven RW, Carvajal TM, Schreier H. Nebulization of liposomes: III. The effects of operating conditions and local environment. *Pharm Res* 1992; 9: 515–20.
87. Elhissi AM, Faizi M, Naji WF, et al. Physical stability and aerosol properties of liposomes delivered using an air-jet nebulizer and a novel micropump device with large mesh apertures. *Int J Pharm* 2007; 334: 62–70.
88. Li Z, Zhang Y, Wurtz W. Characterization of nebulized liposomal amikacin (Arikace™) as a function of droplet size. *J Aerosol Med Pulm Drug Deliv* 2008; 21: 245–54.

17 | Stress testing of combination therapies

Dan W. Reynolds and Biren K. Joshi

INTRODUCTION

This chapter will focus on stress testing of drug products that contain more than one active pharmaceutical ingredient (API). These dosage forms are often referred to as *combination therapies* (1), a special case of *combination products* (2) which usually refer to products consisting of a single API used in combination with a special delivery device and/or a biologic. These dosage forms are also referred to as fixed dose combination (FDC) products and can be formulated as single-compartment FDC or multicompartiment FDC products depending on compatibility and release profiles of individual actives (3). Concerning stress studies of combination therapies, ICH quality guidance Q8 states “for products that contain more than one drug substance, the compatibility of the drug substances with each other should also be evaluated (4).” An FDA guidance states “In many circumstances, the development considerations depend on the type of combination product. When the combination product is comprised of constituents that are chemically, physically or otherwise combined or mixed and produced as a single entity, developers should consider and, as appropriate, evaluate the potential for a broad range of drug/biologic/device interactions (5).” The World Health Organization (WHO) has issued a guideline on submission of generic drugs which states “For fixed-dose combination products, the compatibility of APIs with each other should be studied and the results documented (6).” Clearly, the common theme from these guidance documents is to investigate the possibility of drug–drug reactions in combination therapies.

Most combination therapies involve APIs that are already marketed as monotherapies. Hence, usually there is already a great deal of knowledge available about the degradation chemistry and stability of the individual APIs when formulation development of a combination therapy begins: either in the company that innovated the drugs, the literature, or a pharmacopeia. Consequently, what usually remains to be understood about the new combination therapy from an API stability perspective are potential new drug–excipient reactions and drug–drug reactions (7,8). Associated concerns are development of stability indicating methods (SIMs), adequate packaging, shelf life, and so on.

In this chapter, a brief survey of the literature on stress studies of combination therapies will be given followed by a recommended experimental approach based on the available guidance, the scientific literature, input from the FDA, and successful studies carried out by the authors. Finally, suggestions on what to file in a marketing application will be made.

LITERATURE SURVEY

A critical review of the development of SIMs for drugs made with small molecules was published in 2002 (9). Part of the review focused on stress testing and the development of SIMs for 18 combination therapies. A high-level summary of the samples used for SIM development is shown in Table 1.

As shown in Table 1, there was no consistent approach. The majority of cases (14/18, 78%) did not take into account the possibility of reactions between drug substances. Where stress studies were carried out on individual drugs (entry 2), there was no consistency in what stress conditions (e.g., acid, base, oxidation, etc) were examined or specific conditions (e.g. 50°C or reflux, 0.1N or 1.0N HCl, etc.) used. For example, some workers only examined oxidation while others only acid and base. Only in cases 3 and 4, were drug substances stressed together, these studies tended to use a more comprehensive set of stress conditions.

The author of the review endorsed the use of accelerated and long-term stability samples or stressed product samples for SIM development—instead of samples of the combined APIs stressed under acidic, basic, thermal, oxidative, and photolytic conditions—for combination products containing many active ingredients (some contain 6–10 drugs). The rationale being

Table 1 Approaches to SIM Development for Combination Therapies Reported in Reference 9

Samples Used for Stability Indicating Method Development	Number of Studies
1. Standards of degradation products; no stress studies	5 (28%)
2. Stressed samples of individual drugs only	9 (50%)
3. Stressed drug product	3 (17%)
4. Stressed samples of combined drugs	1 (6%)

that stressed solutions of multiple actives could exhibit high complexity making development of a SIM difficult or impossible. We will return to this topic later.

Our survey (10) of the literature on stress testing of combination therapies is reported in Table 2. The majority (17/29, 59%) of studies referenced were conducted since 2002. Also included are some of the studies reviewed previously. The purpose of most of the studies (24/29, 83%) was SIM development with the remaining aimed at understanding stability of a new formulation. The vast majority (27/29, 93%) involved products containing two APIs with only two products containing three APIs. The dosage forms were tablets (20/29, 69%), capsules (2/29, 7%), a soft gelatin capsule (1/29, 4%), injectables (3/29, 10%), an ophthalmic solution, a rectal gel, and a suspension. The breakdown of what samples were used in the investigations is shown in Table 3.

As shown, 17/29 (59%) studies did address the possibility of drug–drug reactions. Of these, eight stressed the product only, seven stressed individual APIs and product, and two stressed combined APIs. Of the 12/29 (41%) remaining studies where drug–drug reactions were not investigated, nine stressed individual APIs, and three did not conduct stress testing (used standards of degradation products for SIM development). Only one study reported a drug–drug reaction of the 15 investigations where drug–drug interaction was examined. Clearly, the trend in recent years has been toward investigating the possibility of drug–drug reactions albeit in various ways.

Two examples illustrating accelerated degradation of one API in the presence of another in single compartment FDC products have been reported. In the first example, timolol maleate was shown to accelerate the rate of pilocarpine degradation to pilocarpic acid and isopilocarpin in commercial eyedrops (Fig. 1). Pilocarpin eyedrops that did not contain timolol maleate were stable (40). In our view, the enhanced degradation was most likely due to the maleate counter-ion in timolol maleate which catalyzed hydrolysis of the lactone in pilocarpine to pilocarpic acid and its epimerization to isopilocarpine. In the second example, the poor bioavailability of rifampicin was attributed to enhanced degradation of rifampicin under acidic conditions in the presence of isoniazid. Stress-testing studies of the two actives in simulated gastric fluid and 0.1N HCl at 37°C (41) revealed that isoniazid reacted with 3-formyl rifampycin, a degradation product of rifampycine in acid, to alter the kinetics of rifampicin hydrolysis (Fig. 2).

LETTER OF REQUEST FROM THE FDA

As shown in above, many development chemists stress the APIs of combination therapies individually when developing a stability indicating method. However, the obvious question left unanswered by this approach is whether or not the APIs react with each other.

GSK developed a triple combination tablet of the HIV therapies abacavir sulfate, lamivudine, and zidovudine. The three actives were blended together with the excipients in the formulation and were thus in intimate physical contact. Prior to our NDA submission, the FDA wrote us a letter with the following request: “Please conduct one time stress stability studies on a mixture of abacavir sulfate, lamivudine, and zidovudine drug substances. These studies should include acid; base; high temperature and humidity; oxidation; and photolysis

Table 2 Literature on Stress Testing of Combination Therapies

Reference	Purpose of Paper	APIs	Formulation	APIs in Physical Contact in Formulation?	What Was Stressed?	Stress Conditions	Reaction Between APIs	Structures of Degradation Products
11	SIM ^a	Pantoprazole, domperidone	Tablets	Yes	APIs ^b separately, tablets	APIs and tablets: acid, base, peroxide, water, light (solution), dry heat	None reported	For P only
12	SIM	Isoniazid, rifampicin	Tablets	Yes	APIs separately, Tablets	APIs only: acid base, peroxide, light in solution	None reported	None
13	Stability investigation	Artemether, lumefantrine	Tablets	Yes	Tablets	Uncontrolled storage in tropical areas	None reported	None
14	Stability investigation	Amlodipine besylate, atenolol	Tablets, mono and bilayer	Mono yes, bi minimal	Tablets	Accelerated (40C/75%RH) in two packages	None reported	For individual APIs
15	SIM	Amlodipine, benzazepril HCl	Tablets	Yes	APIs separately, tablets	APIs and tablets: acid, base peroxide, solid state dry heat, light in solution and solid state	None reported	None
16	SIM	Valsartan, amlodipine	Capsules	Yes	APIs separately, Tablets	APIs: Acid, base, peroxide, solid state dry heat	None reported	None
17	SIM	Aspirin, warfarin	Tablets	Yes	Tablets	Acid, base, dry heat, heat/humidity, light	Apparent acetyl transfer from aspirin to warfarin	For each API
18	SIM	Atorvastatin, Amlodipine	Tablets	Yes	APIs separately, separate drug formulations	APIs and Tablets: Acid, base, peroxide, solid state dry heat, light in solid state	Not examined	None
19	SIM	Captopril, hydrochlorothiazide	Tablet	Yes	Tablets	Accelerated conditions	None reported	Yes
20	SIM	Cisatracurium besylate, propofol	Injectable	Yes	Combined APIs	Acid, base, peroxide	None	Yes

(Continued)

Table 2 (Continued) Literature on Stress Testing of Combination Therapies

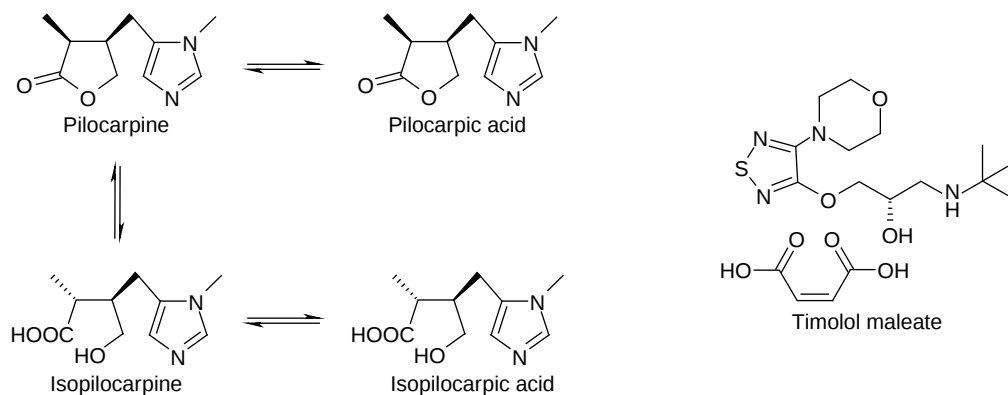
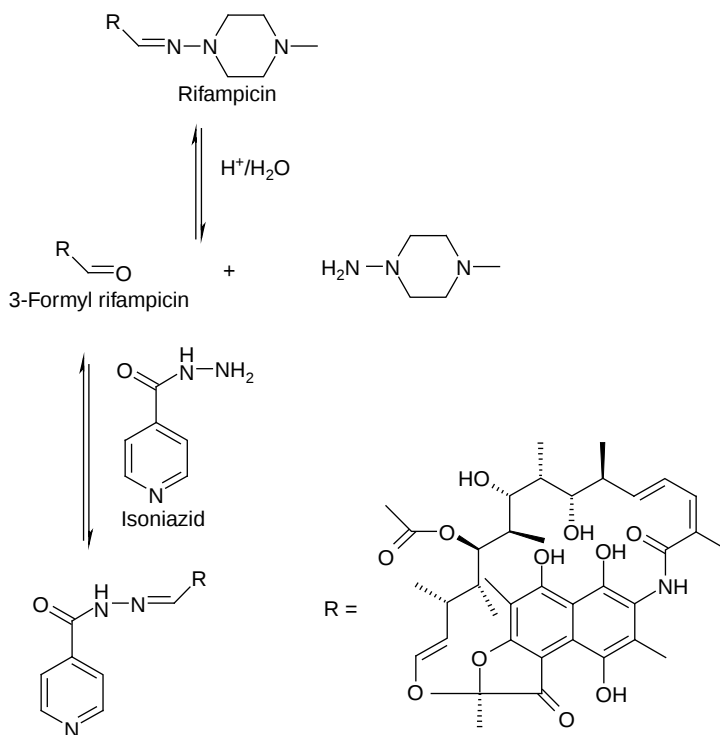
Reference	Purpose of Paper	APIs	Formulation	APIs in Physical Contact in Formulation?	What Was Stressed?	Stress Conditions	Reaction Between APIs	Structures of Degradation Products
21	SIM	Alprazolam, sertraline	Tablet	Yes	APIs separately, tablets	APIs and tablets: Peroxide, acid, base, water, dry heat in the solid state, light in acidic and basic solution and the solid state	None reported	None
22	Stability investigation	Mentronidazole, tetracycline HCl, famotidine	Tablet	Yes	Combined APIs with excipients.	APIs separately and combined in solution at various pH, light, heat; combined drug granules: Heat, heat and humidity, Acid, base, peroxide, solid state: dry heat, heat/RH, light in solution	None reported	Some
23	SIM	Lopinavir, ritonavir	Soft gelatin capsule	Yes	Separate APIs	Heat, heat and humidity, Acid, base, peroxide, solid state: dry heat, heat/RH, light in solution	None reported	None
24	Stability investigation	Ziconotide, clonidine HCl, morphine sulfate	Infusion	Yes	Combined drugs in solution	37C for 7 days	None reported	Some mention of ziconotide pathways
25	SIM	Hydrochlorothiazide, triamterene	Capsule	Yes	Capsules alone and dissolved in solution	Capsules: dry heat; in solution: Acid and base with heat	No	Yes
26	SIM	Hydrocodone bisulfate, acetaminophen	Tablets	Yes	Hydrocodone	Dry heat, acid, base, light in water, peroxide,	None reported	No
27	SIM	Atorvastatin, ezetimibe	Tablets	Yes	APIs separately; tablets	APIs and tablets: acid, base, peroxide, dry heat, light in the solid state	None reported	No
28	Stability investigation, SIM	Losartan, hydrochlorothiazide	Tablets	Yes	Tablets, APIs separately	Tablets: Heat and humidity, acid, base, peroxide; APIs: acid, base, peroxide	None reported	Yes
29	SIM	Naphazoline, tetrahydrozoline	Ophthalmic solution	Yes	Nothing	—	—	Yes
30	SIM	Norgestimate, ethinyl estradiol	Tablets	Yes	APIs separately	Nor: heat and light in the solid state; pH 5, 7, 10 Eth est: heat, humidity	None reported	Yes

31	SIM	Otilonium bromide, diazepam	Tablets	Yes	Nothing	NA	None reported	Yes
32	Stability study	Onansetron hydrochloride and methylprednisolone sodium succinate	Injectable	Yes	Combined injection formulations	Two temperatures	None reported	Unknown-only had abstract
33	SIM	Oxycodone and lidocaine	Rectal gel	Yes	Separate APIs	Hydrogen peroxide at ambient temperature	None reported	No
34	SIM	Pseudoephedrine and cetirizine	Bilayered tablets	Yes	Separate APIs	Acid, base, hydrogen peroxide with heat	None reported	No
35	SIM	Ramipril and hydrochlorothiazide	Various	Yes	Ramipril	Acid and base	None reported	No
36	SIM	Losartan potassium, hydrochlorothiazide	Tablets	Yes	Tablets containing both APIs	Acid, base, peroxide, long term and accelerated stability samples	None reported	Yes
37	SIM	Sulfisoxazole acetyl and erythromycin ethylsuccinate	Suspension	Yes	Nothing; used markers of degradation products	None	None reported	Yes
38	SIM	Tramadol hydrochloride and Aceclofenac	Tablets	Yes	Crushed tablets	Acid, base, H ₂ O ₂ , solid 80°C for 72 hr, and photolysis by exposure to direct sunlight for 72 hr	None reported	None
39	SIM	Telmisartan and Ramipril	Tablets	Yes	APIs separately and crushed tablets	APIs: Acid, base, H ₂ O ₂ , solid 80°C for 28 hr, and photolysis by exposure to short UV (254 nm) for 24 hr and long UV (366 nm) for 48 hr; tablets: centrifuged solutions in Acid, base, H ₂ O ₂ , solid 80°C for 28 hr, and photolysis by exposure to short UV (254 nm) for 24 hr and long UV (366 nm) for 48 hr	None reported	None

^aSIM: Stability indicating method.^bAPI: Active pharmaceutical ingredient.

Table 3 Summary of Approaches to Stress Testing of Combination Therapies in the Literature

Samples Used for Investigation	Number of Studies
1. Standards of degradation products; no stress studies	3
2. Stressed samples of individual drugs only	9
3. Stressed samples of individual drugs and drug product	7
4. Stressed drug product only	8
5. Stressed samples of combined drugs only	2

**Figure 1** Degradation pathways of pilocarpine and the structure of timolol maleate.**Figure 2** Proposed pathway for rifampicin degradation in the presence of isoniazid in acid.

conditions (42).” We complied with that request. In addition, tablets were stressed with heat, heat and humidity, and light.

For the triple combination product, extensive degradation studies on all three individual APIs had already been performed for previous submissions; the major degradation products for each active were known. A single HPLC method was developed with markers of all the significant degradation products plus the three actives (3 APIs + 9 degradation products = 12 compounds). The three APIs were stressed together in solution under acidic, oxidative, and basic conditions and in the solid state with heat, heat and high humidity, and with light. Samples of the combined APIs were stressed until the most labile API under a specific stress condition had degraded 10–20%. The tablets were stressed with heat and heat with high humidity. The HPLC method was used to test the stressed samples of combined APIs and tablets to look for drug–drug reaction products. A drug–drug reaction product was found during the course of these studies which was adequately separated by the method, but was not formed to a significant extent during formal stability studies of the tablets. The results of the degradation studies were reported to the FDA; no questions concerning degradation chemistry were asked.

This approach was also used for advair (fluticasone propionate, salmeterol xinafoate) MDI, combivir (lamivudine, zidovudine) tablets, and treximet (naproxen sodium, sumatriptan) tablets. These studies were also reported in the respective NDAs without questions from regulators.

RECOMMENDED EXPERIMENTAL APPROACH

The recommended experimental approach for stress-testing combination therapies where the APIs are in physical contact in the product will now be described (Tables 4 and 5). Stress studies of APIs usually examine for degradation pathways, mass balance, and stereochemical stability. The degradation chemistry of each active should be established first, either through the literature or from past studies on previous products. If not already done, each API should be stressed individually in the solution (acid, base, oxidation) and in the solid state (heat, heat/humidity, light) (43). These studies will elucidate degradation pathways and provide samples for stability indicating method development. With an understanding of the degradation chemistry of each API in place, studies on the combined actives can begin. It is recommended that a 1:1 molar ratio of APIs be used to maximize potential drug–drug reactions. The solubilities and reactivities of each API should be considered when planning the solution-phase study. With this knowledge, reagent concentrations, use of cosolvents, temperatures, and reaction times can be planned such that the most labile API will be degraded ~10% in a reasonable time (44). This approach is realistic since the same API will be most reactive under formal stability storage conditions, and specifications will likely be based on its behavior. For solid-state studies, APIs can be carefully weighed and combined in vials. Mixing can be achieved by rotating the vial while tilted, with a vortex mixer, or by shaking. After storage, the whole sample is used for testing so mass balance can be assessed. Occasionally, combined APIs may exhibit enhanced hygroscopicity which can impact the chemical stability of the mixture (45). Ideally, one method can be developed that can quantify all APIs and their degradation products. However, different methods for the different APIs may be required. This possibility, in turn, may require multiple sample preparations for analysis. This reality needs to be factored in when planning the stress study. The impurity profiles from the combined stressed actives can then be compared with chromatograms of the important degradation products of all actives obtained with markers or the samples generated from stressing the APIs separately. Any novel peaks in the samples containing the combined APIs can then be interrogated to determine if they are a result of a drug–drug reaction.

The drug product and placebo can be stressed with heat, heat/humidity, and light. The stressed samples can then be analyzed with the appropriate stability indicating method(s). Stressing should be stopped when the most labile API has degraded 10–20% or an appropriate amount of stress has been applied, whichever comes first. Again, comparisons of the impurity

Table 4 Stress Protocol for Multiple APIs in Solution

	Acidic pH	Natural pH/Oxidation	Neutral pH	Basic pH	HPLC Sample Preparation	Tests
Medium	0.1N HCl	Deionized water	pH 7 phosphate 0.1 M	0.1 N NaOH	Prepare [DS] as appropriate: neutralization and dilution	Quantitative HPLC with DAD and MSD detection
[DS]		Typically 0.1–10 mg/mL (APIs in 1:1 mole ratio)				Look for new peaks which might indicate API-API reactions
Organic co-solvents (as needed)	DMSO, acetic acid, propionic acid, CAN	NMP, ACN (10-90% v/v)	NMP, ACN (10–90% v/v)	Glyme, diglyme, dioxane, THF, ACN, DMSO		Peak purity
Headspace	Air, any size	Separate vials of: N ₂ , air, O ₂ headspace (mole O ₂ /DS >100)*	Air, any size	Air, any size		Mass balance Stereochemical stability
Temperature	50–80°C					
Maximum duration	Until at least one API has degraded approximately 10% or assume rate 2× for every 10°C increase; target storage equivalent to 40°C, 6 mo					
Reaction vessel	Sealed clear serum vials or equivalent reaction vessel					

Table 5 Stress Protocol for Multiple APIs in the Solid State

Sample Preparation	Storage Condition	Storage	HPLC Sample Preparation	Tests (As Required)
Each sample for storage should consist of the same number of moles of each API. The samples can be mixed in a vial with a vortex mixer or by simply shaking and/or rotating the vial. A separate sample should be made for each unique storage condition and time point since complete homogeneity cannot be certain. After storage, the entire sample should be tested to facilitate quantitation	Initial	None	Need to do whole sample testing	Quantitative HPLC with DAD and MSD detection
	80°C; 2 wk ^a	Store ~200 mg in a flint glass vial with rubber septum and Al flange collar at 80°C for 2 wk. N ₂ , air, O ₂ headspaces as required	Prepare 0.1 mg/mL [or prepare other (DS) as appropriate] solutions with diluent	Look for new peaks which might indicate API-API reactions Peak purity
	80°C/75%RH: 2 wk	Store 200 mg in an open flint glass vial. Store vial in a jar with saturated brine at 80°C for 2 wk.		Mass balance
	Light ^b Option #1	>2 × ICH conditions (ID65 or D65), ambient temperatures 200 mg spread in a thin layer		Stereochemical Stability
	Light Option #2: Fluorescent then UV	>2 × ICH conditions (ID65 or D65), ambient temperatures 200 mg spread in a thin layer		
	Light Mechanistic Approach: Fluorescent then UV UV	>2 × ICH conditions (ID65 or D65), ambient temperatures 200 mg spread in a thin layer 200 mg spread in a thin layer		

^aSamples also can be stored at 60°C and 60°C/75%RH — 7 wk for retest period purposes and as a backup to the 80°C samples.^bRecommended exposure or about 10% degradation, whichever comes first.

profiles from the stressed product, placebo, and those obtained from the APIs stressed together and separately can then be made to see if novel peaks have formed in the product. LC/MS analysis, and possibly additional studies, should allow for determination of whether novel peaks in the stressed drug product arose from an API, a drug–drug reaction, a drug–excipient reaction, or degradation of an excipient. Isolation and full characterization of novel degradation products may be required if they form in significant amounts (above identification thresholds in Q3B) during formal stability studies.

Not all combination therapies have the APIs in physical contact in the product. For example, dutasteride/tamsulosin hydrochloride capsules consist of a dutasteride soft gelatin capsules and tamsulosin hydrochloride pellets together inside a capsule shell. However, the APIs are not in physical contact, so stress studies were conducted only on the individual product components.

Finally, combination products with many APIs (>5) will be considered. It is apparent from the literature survey and the authors' experience that most combination therapies usually contain 2–3 APIs. Therefore, the protocol described above should be appropriate in most situations. However, where many APIs are involved, the use of stressed samples of combined APIs may afford profiles too complex for SIM development. In these cases, using expired or stressed drug product and placebo samples for SIM development and assessing the formation of new drug–excipient and drug–drug degradation products may be the best option; these samples will contain only the relevant degradation products thereby simplifying method development. It seems reasonable that as the number of APIs increase, the likelihood of finding one SIM for all APIs in the product will decrease.

REGULATORY FILING STRATEGY

As stated before, combination therapies usually consist of APIs that are already in marketed products. In a marketing application, reference can be made to previous submissions where the degradation chemistry of the individual APIs has been reported or the literature if available. SIMs for the respective APIs may have been reported previously. Results of studies on the combined APIs can be reported in the API degradation chemistry module (section 3.2.S.7.3 of the CTD) (46). Recommended contents of this module are listed below.

- Description of stress conditions.
- Scheme of degradation pathways for each API.
- Quantitative results (table) for solution and solid state samples (mass balance).
- Chiral testing results (may refer to previous studies).
- Chromatograms from HPLC testing on key samples.
- Discussion of the formation of each significant degradation product (conditions, mechanism). Include degradation products derived from drug–drug reactions. Dismiss as insignificant peaks observed in stress studies but below Q3A identification thresholds in formal stability studies.
- Summary of peak homogeneity experiments on each API.

Results of studies on the drug product can be reported in the drug product degradation chemistry module (section 3.2.P.8.3 of the CTD) (47). Recommended contents of this module are listed below.

- Description of formulation (potency, excipients).
- Stress conditions.
- Scheme of degradation pathways.
- Quantitative HPLC analysis
- Chiral testing results (may refer to previous studies).
- Chromatograms from analysis.

- Discernment of drug related and excipient only related peaks.
- Discussion of the formation of each significant degradation product (conditions, mechanism). Include degradation products derived from drug–excipient and drug–drug reactions. Dismiss as insignificant peaks observed in stress studies but below Q3B identification thresholds in formal stability studies.
- Summary of peak homogeneity experiments.

If the degradation products observed in the drug product are the same as seen in the stress studies of the APIs, filing a drug product degradation chemistry module may not be required (48).

SUMMARY

Historically most stress studies of combination therapies did not assess the potential for reactions between APIs, but this trend is changing. Regulators suggest examining for potential API–API degradation products. An experimental protocol for stress testing combination products has been presented along with a regulatory filing strategy both of which have been successfully put into practice by the authors. Examining for API–API reaction products can improve analytical methods, lead to more stable formulations, and make products safer.

REFERENCES

1. Searching for articles dealing with stress studies of products with multiple APIs was facilitated by using terms such as “combination therapies” with “forced degradation”, “stress testing”, and “stability”. Similar searches using the term “combination product” were relatively fruitless.
2. Guidance for Industry and FDA Current Good Manufacturing Practice for Combination Products, DRAFT GUIDANCE, September 2004 (<http://www.fda.gov/RegulatoryInformation/Guidances/ucm126198.htm>) (Last accessed October 2009.) See also 21 CFR section 3.2(e),
3. Luo Y, Zu Y, Ahmed SU. Challenges of fixed dose combination products development. *Am Pharm Rev* 2007; 10: 120–6.
4. Guidance for Industry: Q8 Pharmaceutical Development, May 2006.
5. Guidance for Industry and FDA Staff: Early Development Considerations for Innovative Combination Products, September 2006.
6. World Health Organization: Guideline on Submission of Documentation for Prequalification of Multi-source (Generic) Finished Pharmaceutical Products (FPPs) Used in the Treatment of HIV/AIDS, Malaria and Tuberculosis (http://apps.who.int/prequal/info_applicants/Guidelines/GuideGenericSubmitDocFPPs_08_2005_WoAnnexes.pdf) (accessed October 2009)
7. Aubry FA, Tattersall P, Ruan J. Development of stability indicating methods. In: Huynh-Ba, K, ed. *Handbook of Stability Testing in Pharmaceutical Development: Regulations, Methodologies, and Best Practices*. New York, NY: Springer Science and Business Media, 2009: 151.
8. Kazakevich Y, LoBrutto R. *HPLC for Pharmaceutical Scientists*. New York: Wiley, 2007: 497.
9. Bakshi M, Singh S. Development of validated stability-indicating assay methods-critical review. *J Pharm Biomed Anal* 2002; 28: 1011–40.
10. This survey is not exhaustive.
11. Thanikachalam S, Rajappan M, Kannappan V. Stability-indicating HPLC method for simultaneous determination of pantoprazole and domperidone from their combination drug product. *Chromatographia* 2008; 67: 41–7.
12. Ali J, Ali N, Sultana Y, et al. Development and validation of a stability-indicating HPTLC method for analysis of antitubercular drugs. *Acta Chromatograph* 2007; 18: 168–79.
13. Bate R, Tren R, Hess K, et al. Physical and chemical stability of expired fixed dose combination artemether-lumefantrine in uncontrolled tropical conditions. *Malaria J* 2009; 8: 1–7.
14. Aryal S, Skalko-Basnet N. Stability of amlodipine besylate and atenolol in multi-component tablets of mono-layer and bilayer types. *Acta Pharm* 2008; 58: 299–308.
15. Naidu KR, Kale UN, Shingare MS. Stability indicating RP-HPLC method for simultaneous determination of amlodipine and benazepril hydrochloride from their combination drug product. *J Pharm Biomed Anal* 2005; 39: 147–55.

16. Chitlange SS, Bagri K, Sakarkar DM. Asian stability indicating RP-HPLC method for simultaneous estimation of valsartan and amlodipine in capsule formulation. *Asian J Res Chem* 2008; 1: 15–18.
17. Montgomery ER, Taylor S, Segretario J, et al. Development and validation of a reverse-phase liquid chromatographic method for analysis of aspirin and warfarin in a combination tablet formulation. *J Pharm Biomed Anal* 1996; 15: 73–82.
18. Chaudhari BG, Patel NM, Shah PB. Stability indicating RP-HPLC method for simultaneous determination of atorvastatin and amlodipine from their combination drug products. *Chem Pharm Bull* 2007; 55: 241–6.
19. Kirschbaum J, Perlman S. Analysis of captopril and hydrochlorothiazide combination tablet formulations by liquid chromatography. *J Pharm Sci* 1984; 73: 686–7.
20. Zhang H, Wang P, Bartlett MG, et al. HPLC Determination of cisatracurium besylate and propofol mixtures with LC-MS identification of degradation products. *J Pharm Biomed Anal* 1998; 16: 1241–9.
21. Pathak A, Rajput, S Development of a stability-indicating high performance liquid chromatographic method for the simultaneous determination of alprazolam and sertraline in combined dosage forms. *J AOAC Int* 2008; 91: 1344–53.
22. Wu Y, Fassih R. Stability of metronidazole, tetracycline HCl and famotidine alone and in combination. *Int J Pharm* 2005; 290: 1–13.
23. Donato EM, Dias CL, Rossi RC, et al. LC Method for studies on the stability of lopinavir and ritonavir in soft gelatin capsules. *Chromatographia* 2006; 63(9/10): 437–43.
24. Shields D, Montenegro R. Chemical stability of ziconotide–clonidine hydrochloride admixtures with and without morphine sulfate during simulated intrathecal administration. *Neuromodulation: Technol Neural Interface* 2007; 10: 6–11.
25. Menon GN, White LB. Simultaneous determination of hydrochlorothiazide and triamterene in capsule formulations by high-performance liquid chromatography. *J Pharm Sci* 1981; 70: 1083–5.
26. Wallo WE, D'Adamo A Simultaneous assay of hydrocodone bitartrate and acetaminophen in a tablet formulation. *J Pharm Sci* 1982; 71: 1115–18.
27. Chaudhari BG, Patel NM, Shah PB, et al. Stability-indicating reversed-phase liquid chromatographic method for simultaneous determination of atorvastatin and ezetimibe from their combination drug products. *J AOAC Int* 2007; 90: 1539–46.
28. Lusina M, Cindric T, Tomaic J, et al. Stability study of losartan/hydrochlorothiazide tablets. *Int J Pharm* 2005; 291: 127–37.
29. Bauer J, Krogh S. High-Performance liquid chromatographic stability-indicating assay for naphazoline and tetrahydrozoline in ophthalmic preparations. *J Pharm Sci* 1983; 72: 1347–9.
30. Lane PA, Mayberry DO, Young RW. Determination of norgestimate and ethinyl estradiol in tablets by high-performance liquid chromatography. *J Pharm Sci* 1987; 76: 44–7.
31. Mannucci C, Bertini J, Cocchini A, et al. High-performance liquid chromatographic method for assay of otilonium bromide, diazepam, and related compounds in finished pharmaceutical forms. *J Pharm Sci* 1993; 82: 367–70.
32. Bougouin C, Thelcide C, Crespin-Maillard F, et al. Compatibility of ondansetron hydrochloride and methylprednisolone sodium succinate in multilayer polyolefin containers. *Am J Health-System Pharm* 2005; 62: 2001–5.
33. Gebauer MG, McClure AF, Vlahakis TL. Stability indicating HPLC method for the estimation of oxycodone and lidocaine in rectal gel. *Int J Pharm* 2001; 223: 49–54.
34. Makhija SN, Vavia PR. Stability indicating HPTLC method for the simultaneous determination of pseudoephedrine and cetirizine in pharmaceutical formulations. *J Pharm Biomed Anal* 2001; 25: 663–7.
35. Belal F, Al-Zaagi IA, Gadkariem EA, et al. A stability-indicating LC method for the simultaneous determination of ramipril and hydrochlorothiazide in dosage forms. *J Pharm Biomed Anal* 2001; 24: 335–42.
36. Hertzog DL, McCafferty JF, Fang X, et al. Development and validation of a stability-indicating HPLC method for the simultaneous determination of Losartan potassium, hydrochlorothiazide, and their degradation products. *J Pharm Biomed Anal* 2002; 30: 747–60.
37. Elrod L, Cox RD, Plaszc AC. Analysis of oral suspensions containing sulfonamides in combination with erythromycin ethylsuccinate. *J Pharm Sci* 1982; 71: 161–6.
38. Kachhadia PK, Doshi AS, Ram VR, et al. Validated LC method for simultaneous analysis of tramadol hydrochloride and aceclofenac in a commercial tablet. *Chromatographia* 2008; 68: 997–1001.
39. Patil KR, Rane VP, Sangshetti JN, et al. A stability-indicating LC method for the simultaneous determination of telmisartan and ramipril in dosage form. *Chromatographia* 2008; 67: 575–82.

40. (a) Pilatti C, Torre M, Chiale C, et al. Stability of Pilocarpine Ophthalmic Solutions. *Drug Dev Ind Pharm* 1999; 25: 801–5. (b) The mechanism of Pilocarpine degradation has been studied extensively. See Klaus, ed. *Analytical Profiles of Drug Substances*. Vol. 12. New York: Academic. 1983: 385
41. Singh S, Mariappan TT, Sharda N, et al. The reason for an increase in decomposition of rifampicin in the presence of isoniazid under acid conditions. *Pharm Pharmacol Commun* 2000; 6: 405–10.
42. This request was received June 9, 1999 concerning the April 20, 1999 investigational new drug (IND) application for Triple Combination Tablets.
43. Experimental conditions for stressing combined APIs can be essentially the same as used for individual APIs. For full stress testing protocols see (a) Reynolds DW. Forced degradation of pharmaceuticals. *Am Pharm Rev* 2004; 7: 56–9. (b) Jansen PJ, Smith WK, Baertschi SW. Stress testing: analytical considerations. In: Baertschi SW, ed. *Pharmaceutical Stress Testing*. Boca Raton, FL: Taylor & Francis, 2005: 141–71.
44. In some cases, the APIs may be exceptionally stable and not degrade much even after days under extreme conditions. In these cases, storage for 2 weeks at 80°C or 6 weeks at 60°C should suffice, even if there has been little degradation. These conditions are based on the assumption that the reaction rate doubles for every 10°C temperature increase and that the target is to equal storage at 40°C for 6 months, a typical accelerated storage condition. See citations in reference 43 for more information.
45. Salameh AK, Taylor LS. Role of deliquescence lowering in enhancing chemical reactivity in physical mixtures. *J Phys Chem B* 2006; 110: 10190–6.
46. Guidance for Industry: M4Q: The CTD - Quality, CDER, 2001 (ICH) (<http://www.fda.gov/downloads/RegulatoryInformation/Guidances/UCM129904.pdf>) (last accessed October 2009)
47. See reference 46.
48. One company asked the FDA: “Do CGMPs require that forced degradation studies always be conducted of the drug product when determining if a drug product stability test method is stability-indicating?” The answer was “no”. See <http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm124785.htm#2> (last accessed October 2009).

18 | **Rapid stress stability studies for evaluation of manufacturing changes, materials from multiple sources, and stability-indicating methods**

Bernard A. Olsen, Michael A. Watkins, and Larry A. Larew

INTRODUCTION

Frequently, time is of the essence. It is necessary to evaluate, in a short time span, the stability of a product change, and here, the design of stress conditions which will accelerate decomposition in a meaningful way is necessary. This is a difficult task and one very particular to the stability scientist.

—J. T. Carstensen (1)

Understanding the stability characteristics of drug substances and drug products is a critical activity in drug development and the above quote by Carstensen underscores the need to learn of potential stability problems as soon as possible during development. It is also necessary to evaluate potential stability differences for drug substance starting materials and intermediates prepared by different routes or by different suppliers. In early phases of development, testing drug substances or products held at conditions to induce degradation (stress conditions) is often performed to gain an understanding of the drug's inherent stability and to identify degradation products and pathways. Additional studies may also be conducted to support the packaging materials used to store products. Stability studies under normal storage and accelerated conditions are conducted to support use of the drug during clinical trials and ultimately for marketing applications (2). Accelerated conditions used for these purposes may or may not degrade the samples. Definitive studies on the drug substance from the commercial synthesis and the drug product in the final market formulation including packaging are necessary for product registration. Results from these studies are used to establish appropriate specifications and to justify product dating. Requirements for such studies are described in the International Conference on Harmonization (ICH) guidelines for stability (3).

Stability-indicating analytical methods are fundamental to the evaluation of the stability characteristics of synthetic starting materials, intermediates, drug substances, and drug products. Rapid development of these methods, including data to support their stability-indicating capability, is needed for early and later stages of drug development.

As stability information becomes established it serves as a comparison for future development. If production process, formulation, or packaging changes during or after development are contemplated, the effect of the change on stability must be evaluated. This is especially important for compounds or formulations that are relatively labile. Regulatory guidelines describe stability studies needed for post-approval changes, where the definitive stability has already been established (4). In most cases, a minimum of a study under accelerated conditions for 3 months and a concurrent room temperature study are needed to support changes with the potential to affect stability behavior. Before the resources and time necessary to produce material and conduct such studies are committed, it is valuable to have an indication that the studies will be successful, that is, that the change will not have an adverse effect on stability. A rapid indication of stability impact is also helpful in guiding additional development or optimization work.

The use of accelerated/stress conditions beyond those used for normal 3–6 month studies can provide an indication of relative stability behavior when comparing one sample to another. Equivalent behavior under these conditions will be an indication, if not guarantee, that the materials will behave similarly under less stringent conditions but longer times.

Comparative studies may also be useful in evaluating the relative stability of different commercial formulations of the same drug from different suppliers. In these cases, the cause(s) of stability differences may be difficult to determine since different sources of active ingredient and different excipients may all be variables that affect stability. Changes in the source of drug substance or excipient, or changes in the primary package components could also be evaluated individually by holding other variables constant.

In this chapter, considerations for conducting comparative stress stability studies are described. Although the detailed examples are primarily related to drug substance supply, the principles outlined here can be applied to excipient, process and package component changes, as well. Examples are presented to illustrate considerations and approaches for using stress conditions to compare sample stability. Discussion and an example of synthetic starting material stability evaluation are also included since many of these materials may be obtained via multiple synthetic routes and/or multiple suppliers. A related approach using statistical modeling is described, whereby the stability of a labile compound is modeled as a function of moisture and temperature. While requiring more time than some stress condition studies, this approach allows prediction of stability behavior over a wide range of conditions and ultimately saves time by removing the need for additional studies. Finally, a one-day stress protocol is described which can rapidly generate samples that can be used to develop analytical methods or to demonstrate the stability-indicating capability of a method.

CONSIDERATIONS FOR COMPARATIVE STRESS STABILITY STUDIES

Much work has gone into conducting accelerated studies as a faster means of predicting normal storage temperature degradation rates and justifying expiration dating (5–17). That is not the purpose of the approach described here. Instead, the goal here is to design rapid studies capable of showing stability differences among samples that may indicate differences under normal conditions. Likewise, if no differences are observed, more confidence is gained that the materials will display equivalent behavior under normal conditions.

Stress conditions are often considered to be more severe than accelerated conditions used for marketing application stability studies. For example, ICH stability guidelines state that stress testing should include “the effect of temperatures (in 10°C increments (e.g., 50°C, 60°C) above that for accelerated testing), humidity (e.g., 75% relative humidity or greater) where appropriate, oxidation, and photolysis on the drug substance” (3). The stress conditions are usually chosen to induce degradation in an amount of time that the investigator deems appropriate. This is always a compromise between obtaining results in a short time by using more extreme conditions versus producing degradation that is not representative of pathways operative at less extreme conditions.

Considerations involved in planning, conducting, and interpreting comparative stress stability studies are discussed below. The purpose of the study, e.g., comparing excipient source, process change, or package component, should also be considered when designing the experimental plan.

1. *Previous knowledge:* Although it may be obvious, information such as degradation product identity, degradation pathways, stability under various conditions, and the ability of analytical methods to determine degradation products is very valuable. Without this information, more extensive preliminary experiments will be required to understand degradation behavior and aid in the choice of appropriate stress conditions.
2. *Representative degradation:* Stress conditions which produce a degradation profile similar to that observed under normal conditions should be used. Production of different degradation products indicates that a different degradation pathway is operative and results may not reflect comparative room temperature stability. Investigators are often cautioned against extrapolating too far between stress/accelerated and normal conditions (18). Problems with extrapolations from Arrhenius studies (19,20), with the importance of controlling

relative humidity (16,17), and with changes in the degradation mechanism of proteins as a function of temperature are examples that have been noted (21–23). Also, conditions that are too harsh may produce degradation rates that do not discriminate stability differences among samples, whether or not they would display differences under normal conditions. Conditions that are too mild, however, will require excessive time to observe differences and therefore defeat the purpose of a rapid stability comparison. Samples should also be studied in the state in which they normally exist, e.g., solution studies should not be used to compare stability of solid-state materials.

When comparing drug products containing different formulation components, different impurities may arise due to drug–excipient interactions. In these cases, comparative stress testing begins to overlap with the concept of drug–excipient compatibility studies.

3. *Extent of degradation:* Degradation levels of interest will vary depending on the compound and purpose of the study. Time/temperature combinations that produce levels of degradation products or loss of potency that would cause failure to meet specifications would be reasonable to use for comparative stress studies.

For very stable drugs, the utility of stress comparison studies may be to show that manufacturing or formulation changes have not caused a change that could impart instability such as the presence of greater amorphous content. Showing good stability under stress conditions would add confidence that the change did not affect stability properties.

4. *Analytical method:* The method used to detect degradation must be suitable for its intended purpose. Loss of potency of the drug is often the measured response for stability studies. This may reflect a lack of specification limits and methodology for impurities in pharmacopeial monographs, especially for dosage forms and older drug substances. It is also sometimes assumed that monitoring degradation products is not necessary since it will only mirror loss of potency.

Analytical precision relative to the amount of degradation is the primary reason that determination of degradation products rather than loss of potency is recommended (16,24–26). Potency results have been used when degradation well beyond the levels considered pharmaceutically acceptable was studied, usually in connection with mechanistic determination. For more relevant amounts of degradation (~2–5%), the inherent variability of most potency measurements (~1%) is on the order of the differences of interest among samples. Therefore, many assay replicates would be needed to reduce measurement variability to acceptable levels. Measurement of degradation products using methods such as high-performance liquid chromatography (HPLC) can typically provide much better precision relative to the changes being investigated.

Isothermal calorimetry is also a technique that may provide a rapid comparison of stability among different samples (27,28). Minute quantities of heat produced as a compound degrades even at normal storage temperatures can be determined and degradation rates projected. Disadvantages to this approach are the need for instrumentation that is not available in many laboratories and the inability to distinguish between degradation and other thermal events such as relaxation of a “higher energy” crystal lattice and other physical changes.

5. *Humidity control:* Moisture is an important factor in the stability of many pharmaceutical compounds and formulations (16,17,29,30). The decision to control moisture for comparative stress studies should depend on the characteristics of the compound and the information desired. The moisture content of typical samples, moisture sorption as a function of relative humidity, and the effect of moisture on degradation can help guide the decision to control humidity. As a general practice, samples being compared should be exposed to the same ambient humidity conditions in the storage chamber.
6. *Packaging:* Unless different packaging materials or systems are being compared, the same packaging should be used for comparative stress studies. The packaging options also include “open dish” conditions where no packaging is used. The relative stabilities of the drug substances or products stored under similar conditions can then be ascertained.

7. *Determination of differences in results:* The significance of an observed difference in degradation rates between samples must be known to determine whether the difference is within experimental error or due to different stability properties of the samples. Checking several containers of the same sample under the stress conditions can be used to establish the reproducibility of the observed degradation rate. This would include variability due to the individual samples and the measurement method. Differences greater than this or some higher value set considering the desired confidence level could then be attributed to stability differences. Confirmatory data (see below) are also very useful in establishing the magnitude of difference under stress conditions that can reflect room temperature differences. If comparisons of samples not stressed at the same time are desired, a further evaluation of study-to-study variability might be required. In most cases, a direct comparison where the samples to be compared are all treated in the same study is recommended.
8. *Kinetic model/Arrhenius study:* Various kinetic models have been proposed for solid-state degradation (17,31). Degradation plots for several models are given in Figure 1. Most of these models display approximately linear or zero-order behavior in the pharmaceutically relevant range of about 10% degradation. An exception is the Avrami–Erofeev model using an exponent of less than about 0.5 which starts to show a sigmoidal degradation pattern with an initial lag phase. For most purposes, a zero-order model provides a simple and convenient means of data comparison for stress studies. Also, accurate kinetic modeling is usually not the purpose of stress studies and becomes even more tenuous for solid dosage forms containing mixtures of compounds. If nonlinear degradation data are obtained, other models can be used if fitting data to obtain a rate constant is desired. For most development purposes, a strictly empirical rather than theoretical or mechanistic approach is adequate to compare the relative stability of samples. For some studies, only initial and final time point measurements may be adequate. The number of time points should be increased for more

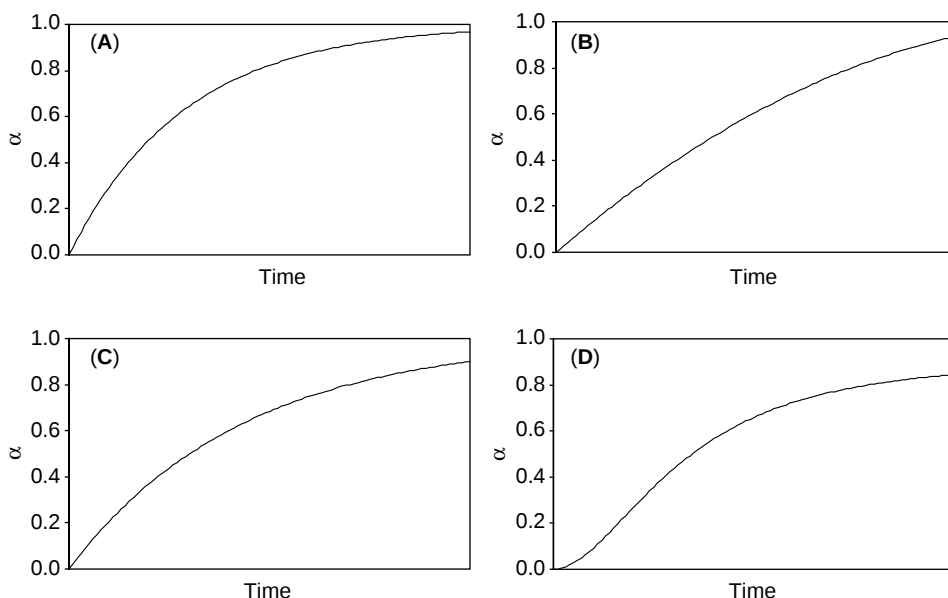


Figure 1 Solid state degradation models. α = fraction decomposed. A = Prout–Tompkins $kt = \ln\left(\frac{1}{1-\alpha}\right)$; B = 2 dimensional phase boundary $kt = 1 - (1 - \alpha)^{1/2}$; C = Avrami–Erofeev, $kt = [-\ln(1 - \alpha)]^n$, $n = 1$; D = Avrami–Erofeev, $n = 0.5$.

accurate determinations of rate constants if desired or needed. Four points are usually sufficient for linear degradation.

For greater confidence that stress conditions are predictive of relative stability under normal conditions, an Arrhenius study of degradation rate at different temperatures can be performed and a room temperature rate calculated. Although a study with three temperature points can be used, four points provide greater confidence in the fit to the Arrhenius equation (7). Knowledge of the observed degradation rate at room temperature from previous studies is necessary for comparison. A predicted rate that is consistent with the observed rate can provide good evidence of the applicability of the stress conditions for predicting room temperature behavior.

9. *Confirmatory studies:* In addition to consistency of Arrhenius predictions with observed room temperature data, it may be desirable to check the agreement of results from comparative stress studies with results at room temperature. This provides a degree of validation that the stress conditions will provide results predictive of real differences observed at lower temperatures over a longer period of time. Since the room temperature data will take longer to obtain, this type of confirmation may be obtained after initial stress comparison studies are well over. Consistency of results will provide additional confidence for future studies, however.

LITERATURE EXAMPLES

Accelerated or stress stability studies for the purpose of predicting sample shelf life at normal conditions have been investigated for many years. In addition, some examples of stress stability studies for sample comparison have been described. Goldberg and Nightingale (32) compared the stability of aspirin in a combined dosage form with propoxyphene. The hydrolysis product, salicylic acid, was monitored in samples stored at 25°C, 37°C, and 50°C at both low (<10%) and high (90%) relative humidity. Data were reported for up to 28 days. One formulation showed instability at room temperature in this time, while the others were stable. At 50°C, different degradation rates were observed for the three samples with the fastest rate obtained for the sample that degraded at room temperature. The degradation rate differences were apparent within 2 weeks at 50°C. The enhanced stability of one sample was postulated as due to physical separation of propoxyphene from the aspirin in pelleted form. This separation may have prevented exposure of the aspirin to an acidic environment caused by contact with the HCl counter ion of propoxyphene.

Furlanetto et al. (33) attempted to predict and compare the solid-state stability of cefazolin sodium and cephaloridine sterile powders using data obtained at 37°C, 45°C, and 60°C. They analyzed samples for up to 6 months and found cephaloridine to be more stable than cefazolin sodium, in contrast to the relative stability of the compounds as indicated by their respective compendial storage instructions. They attributed greater stability of the cephaloridine samples to greater crystallinity compared to the cefazolin sodium samples which appeared to be mostly amorphous. Rather than comparing different samples of the same drug, this study determined the relative stability of two different drugs and compared the results to expectations based on previous information.

An example of the use of accelerated conditions during development is given by Barthomeuf et al. (34) who compared the stability of vapreotide (an octapeptide somatostatin analog) freeze-dried preparations with lactose and glutamic acid/sodium glutamate as stabilizing agents. Studies were conducted at 50°C, 70% RH for 3 weeks, with HPLC monitoring of vapreotide content and levels of degradation products. The glutamate buffered formulation was more stable. The lactose formulation showed evidence of Maillard reactions leading to degradation in addition to oxidative and peptide bond-breaking mechanisms.

Felton et al. (35) described a rapid technique utilizing differential scanning calorimetry for the comparison of oxidative stability among different samples. Samples were heated to a specific temperature (110, 120, or 125°C) under nitrogen and then exposed to oxygen while

holding the temperature constant. Oxidative induction times were measured from the real-time heat flow graphs. The effectiveness of different antioxidants such as BHA, BHT, and ascorbic acid at different levels in an oxygen-sensitive drug could be compared in a short time for rapid screening of solid-state stability.

Concerns about degradation as a result of transporting products under uncontrolled conditions have been raised, particularly in regard to mail-order distribution and actual conditions of use by patients. For example, Black and Layloff (36) showed that mailbox temperatures could reach values as high as 58°C. Zahn has shown that products being shipped globally can reach temperatures up to 70°C for a couple of hours (see chap. 23). Temperature cycling stability studies have been proposed to mimic temperature ranges that might be experienced during product distribution (37). Accelerated conditions have been used as a means of identifying labile products which might require greater control during distribution (38–41). In these studies, the effect of direct exposure (open-dish) to temperature/humidity combinations (30°C/75% RH or 25°C/60% RH) on properties such as appearance and dissolution was determined. The results gave an indication of products that might need special labeling or shipping requirements.

Waterman and Adami discussed several aspects of the use of accelerated conditions for the prediction of drug stability and provided cautions related to Arrhenius-based extrapolations from accelerated to normal conditions (16). Nonadherence to Arrhenius behavior over a wide temperature range could be caused by phase transitions, pH shifts, uncontrolled relative humidity, complex reaction mechanisms, and changes in Arrhenius parameters with temperature. In particular, the importance of water activity and relative humidity for conducting accelerated studies was described.

Waterman et al. (17) developed a protocol and data analysis to obtain a rapid estimate of shelf-life of solid oral dosage forms using accelerated conditions. Instead of conducting stability studies for a set time period, they used an isoconversion endpoint where studies are continued until a set level of degradation is reached. This compensates for heterogeneous kinetics in the solid state that may be due to crystalline defects or amorphous material. They also employed a moisture-corrected Arrhenius equation to compensate for the effect of relative humidity on reaction rates. A two-week study at 50°C/75% RH, 60°C/45% RH, 70°C/75% RH, and 80°C/5% RH was proposed for early-phase excipient compatibility screening. Uncertainty of predicted rates could be determined using statistical analysis of error propagation. These concepts were also discussed in a recent book chapter (42).

A model based on moisture increase in a drug product package and hydrolytic degradation rates predicted from Arrhenius studies was used to predict degradation rates in various packaging designs (43). Water vapor permeability rates, initial moisture levels, tablet and desiccant moisture sorption isotherms, degradation rate, and temperature were used to model hydrolysis under varied conditions. Good agreement of models with experimental data was obtained.

Detailed Example—Cefaclor Monohydrate

Most of the considerations given for comparative stress studies are illustrated in an investigation of the relative stability of cefaclor monohydrate drug substance and capsule formulations (44). Differences in the levels of degradation products present in several commercial cefaclor products with similar expiration dates had been noted. The goal of the investigation was to quickly determine if some cefaclor capsule formulations were less stable than others without performing long-term stability studies on each product.

1. *Previous knowledge:* A fairly extensive description of cefaclor degradation was available in the literature including methodology for determination of degradation products (45) and identification of degradation products and pathways under different conditions (46).
2. *Representative degradation:* Figure 2 shows the qualitative degradation product profiles obtained for cefaclor monohydrate drug substance after preparation, held at room

temperature for 2.4 years, and held at 67°C for 2 weeks. The major degradation products and nearly all the minor products in the 2-year and 2-week heated samples are identical, thereby providing confidence that degradation pathways at 67°C are the same as those at room temperature.

3. *Degradation level:* The limit on total related substances in cefaclor monohydrate given by the United States and European Pharmacopeia is 2.0%. This establishes a level of degradation that would be considered significant. For the drug product, degradation of 10% would cause the potency to fall below the pharmacopeial minimum of 90% assuming that no overage was used initially. Taking these as benchmarks for significance, the stress conditions should target degradation levels from 2 to 10%.
4. *Analytical method:* Monitoring degradation product levels would provide a better indication of stability differences than a stability-indicating potency method. The relative change in degradation levels could be several fold while the potency change could be as little as 2%, which is within the variability of the potency assay and capsule content uniformity. An HPLC method for related substances was therefore used to assess the amount of degradation.
5. *Humidity control:* The question of humidity control was examined in several ways. The moisture sorption isotherm showed moisture levels from 3 to 6% over a relative humidity range of 20–95%. Also, samples held at 65°C for over 2 weeks remained within this range of water content which was also within pharmacopeial specification. Finally, samples which were heated did not show a change in crystal form by X-ray powder diffraction, such as partial conversion to an anhydrate. Based on these results, humidity control was not used for comparative stress studies. Samples to be compared were all stored under identical conditions, i.e., in the same oven. Any local change in humidity around each sample was therefore a function of the sample itself.
6. *Packaging:* Most commercial samples were available in plastic bottles so similar bottles were used for comparative studies. The same type of bottle was used for all samples to remove packaging as a variable.
7. *Significant differences:* For cefaclor monohydrate capsule formulations, the average standard error in determination of the slope of the degradation plot was 0.012 %/day for degradation rates covering a range of 0.04–0.22 %/day. A difference of about 0.04% (three times the standard error) in the rates could indicate a significant difference in sample stability. Rate differences for the same samples obtained in different studies were 0.02, 0.01, and 0.06 %/day for three different lots of material. The 0.06 %/day difference was observed for a less

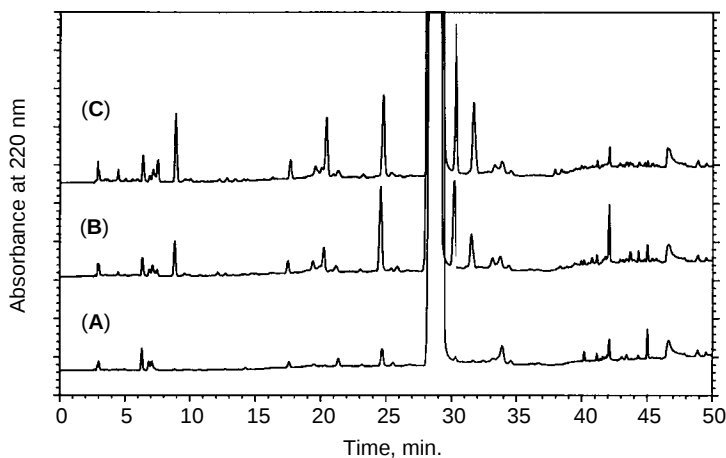


Figure 2 Degradation product profiles of cefaclor monohydrate: (A) 1 month after preparation; (B) after 2 weeks at 67°C; (C) after 2.4 years at room temperature.

stable sample (degradation rate = 0.2 %/day) while the other differences were for samples showing lower rates (degradation rate = 0.1 %/day). Also, as discussed below, confirmatory data showed that samples with a significant room temperature stability difference showed a stress difference of 0.08 %/day, which is greater than the significance threshold identified above.

8. *Kinetic model/Arrhenius study:* Preliminary studies showed that most samples degraded in a linear fashion, which would suffice for comparing degradation rates. A more detailed kinetic model was not needed but an Arrhenius study was conducted to check the agreement of the predicted room temperature degradation rate with the observed rate. Degradation rates were determined for cefaclor monohydrate stored at 45, 55, 64, and 70°C for a period of 2 weeks. Samples were held in closed vials with uncontrolled humidity. Water content reflected no change from the monohydrate. This encompassed the temperature (65°C) being considered for comparative studies. The increase in degradation products versus time for these conditions is shown in Figure 3. Zero-order degradation rates were taken from the slopes of the regression lines which included measurements at a minimum of four time points. The 45 and 55°C conditions produced increases in degradation of less than 1% showing that much longer times would be necessary to obtain degradation of at least 2%. As observed with other preliminary studies, storage at 65°C for about 2 weeks degraded samples by about 2%. Even though rates were faster at higher temperature, two weeks was a reasonable time to conduct studies without increasing the risk of nonrepresentative degradation at higher temperatures.

The Arrhenius plot from the degradation rates obtained in Figure 3 gave a slope of -8462 with a coefficient of determination (r^2) of 1.000. The extrapolated degradation rate at 20 °C was 1.1%/year with a 99% confidence range of 0.6–2.2%/year. The observed degradation rate over a period of 2 years at room temperature was 0.6%/year. This is at the lower end of the range from the Arrhenius study and provides additional confidence that comparing relative stabilities at 65°C is likely to reflect their behavior at room temperature. The difference between the Arrhenius and observed rates also illustrates the danger in using Arrhenius studies alone to determine expiry dating.

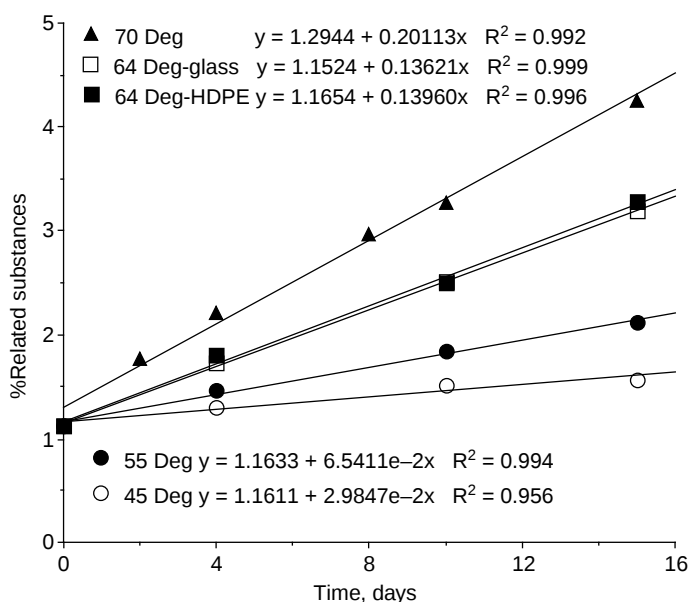


Figure 3 % Degradation versus time at different temperatures. Data at 64°C were obtained in glass and high-density polyethylene (HDPE) bottles.

9. *Confirmatory data:* Two samples that had shown differences in stability at 25 °C were compared under stress conditions. The degradation rates were 0.17 and 0.25%/day for the two samples. This was a significant difference given a standard error of about 0.01–0.02%/day in the rates and showed at least in one case, the ability of the stress results to reflect stability differences at room temperature.

The stability of capsule formulations was also compared under stress conditions. As with the drug substance, degradation was usually linear and the rates provided a basis for comparison. Sample degradation rates could be generally classified as $\leq 0.11\%$ /day or $\geq 0.16\%$ /day to distinguish materials with different stability behavior. This was based on the reproducibility of determining degradation rates and room temperature data showing less stability among samples in the higher rate group compared to samples with lower stress degradation rates. Greater uncertainty would accompany conclusions about the relative stability of samples falling within the same group.

Some capsule formulations did not show linear degradation under stress conditions. A rapid rate was observed initially which then decreased and became more linear. The presence of rapidly degrading amorphous material was investigated and shown to be a possible explanation for this behavior. This behavior might also be caused by consumption of low-level impurities mediating degradation, but this was not investigated. Differences in the shape of stress degradation versus time curves could also be indicative of other chemical or physical differences among samples.

Conclusions from these studies were that stability studies at 65°C for 2 weeks could be used to compare the stability characteristics of cefaclor monohydrate samples, either as bulk or in capsule formulations. Observations of differences could be used as an alert that further investigation was needed if a manufacturing or supplier change was being considered. Differences in relative stability of commercial formulations could explain differences in quality observed in the marketplace or in development. Decisions about the need and type of further investigations are greatly facilitated by obtaining information in only 2 weeks.

STATISTICAL DESIGN STUDIES

In some development projects, knowledge of stability behavior over a wide range of conditions is desired. Various combinations of temperature and humidity to which a drug substance or product may be exposed are often concerns for relatively labile materials. It is very impractical to compare different samples under all conditions of interest, but statistical experimental design offers an efficient means of developing a predictive model for stability under a variety of conditions. This type of program will usually require a more significant time and resource investment than the comparative studies described previously but can ultimately save time by directing development work toward conditions that will ensure adequate stability. Statistical design studies may also be useful for screening to identify parameters that affect stability and to help optimize the stability of a formulation. While formulation optimization is often the goal of these types of studies, examples are included here since accelerated or stress conditions are usually employed in such studies to compare many conditions in one set of experiments.

Statistical design of experiments is commonly used for optimization problems (47). Jones (48) has provided an overview of response surface methodology in the context of stability. Remunan et al. (49) used a factorial design to study the effect of tableting force (0, 6000 N, 12,000 N), relative humidity (0% and 80%), and temperature (20 and 40°C) on the chemical stability and dissolution behavior of sustained release nifedipine tablets. Response surfaces demonstrated the significant impact of relative humidity on dissolution behavior over time and showed the need to protect the formulations from moisture. Tablet compression force also affected dissolution. Nguyen et al. (50) used a central composite design to study the effect of pH, sucrose, propylene glycol, glycerin, and EDTA, each at three levels, on the chemical stability and preservative efficacy of lamivudine liquid formulations. Samples were held at 30°C and

40°C for 3 months. The pH of the formulation was found to be the main factor influencing stability of the drug and preservative. Bos et al. (51) described factorial designs to assess the effect of variables such as disintegrant concentration, compression load, storage temperature, and storage humidity on the physical stability of tablets as indicated by crushing strength and disintegration time. All four variables affected the responses observed, and the ratio of a response after storage to the initial response was determined to be a useful means of comparing different batches of tablets.

A detailed example follows for a statistically designed study to determine the effect on stability of temperature, relative humidity, and the water content of a drug substance under development in the authors' laboratory. The drug substance was obtained initially as an amorphous form, but modifications made to the method of isolating the drug substance appeared to result in greater crystallinity and improved stability properties. More detailed information concerning the stability behavior of both forms over a range of temperature and moisture content was desired.

The experimental design is given in Table 1. This is a full factorial design with duplicate center points. Of course many other statistical designs are possible depending on the information desired, degree of precision desired, and resources available to execute the protocol. The experimental design used is usually a compromise between the completeness or precision of the results and the analytical resources required to conduct the study. Fractional factorial designs may be used to study more variables while keeping the number of experiments manageable. It is beyond the scope of this discussion, but an important consideration in fractional factorial designs is the limitations of information on variable effects and their interactions that may be obtained. For example, screening designs such as fractional factorials are useful in identifying main effects, but some variables and variable interaction terms may be confounded, so care should be taken in interpreting the results.

As indicated from moisture sorption data, the water content of the samples was a function of relative humidity. Storage conditions of 0, 45, and 75% relative humidity were used to control water content of the samples at approximately 3, 6.5, and 10 wt/wt%, respectively. The consistency of these values was checked at each stability time point.

Levels of three individual degradation products and the total amount of degradation impurities were monitored at time points of 1, 2, 4, 8, 12, and 24 weeks. Statistical software was used to analyze the data and derive models for the rates of degradation as a function of the temperature and sample water content. The analysis showed that temperature and water content were both significant in their effect on degradation rate. The interaction term water*temperature and the curvature term temperature*temperature were also statistically significant.

Parameter estimates for the statistical models were used to generate response surface maps for both the amorphous and crystalline forms (Figs. 4 and 5, respectively). These maps are very useful for assessing and comparing the predicted stability properties of the two forms

Table 1 Experimental Design for Stability of a Development Compound:
For Temperature, -1 = 5°C, 0 = 25°C, 1 = 45°C; For Water Content, -1 = 3%,
0 = 6.5%, 1 = 10%

Experiment Number	Temperature	Water Content
1	-1	-1
2	1	-1
3	0	0
4	-1	1
5	1	1
6	0	0

under a range of temperature and humidity (water content) conditions. The amorphous form was stable over a much smaller temperature–humidity region than the crystalline form. The stability of the amorphous form was particularly sensitive to humidity. The crystalline form could tolerate moisture much better as long as the sample was held at an appropriate temperature. The model shows which combinations of temperature and moisture content will provide good stability.

Similar analyses for individual degradation products reveal interesting behaviors. For example, the pattern for peak B for the amorphous form (Fig. 6) is similar to the total related substances' results. In contrast, the rate of peak F formation for the crystalline form is almost independent of water content (Fig. 7).

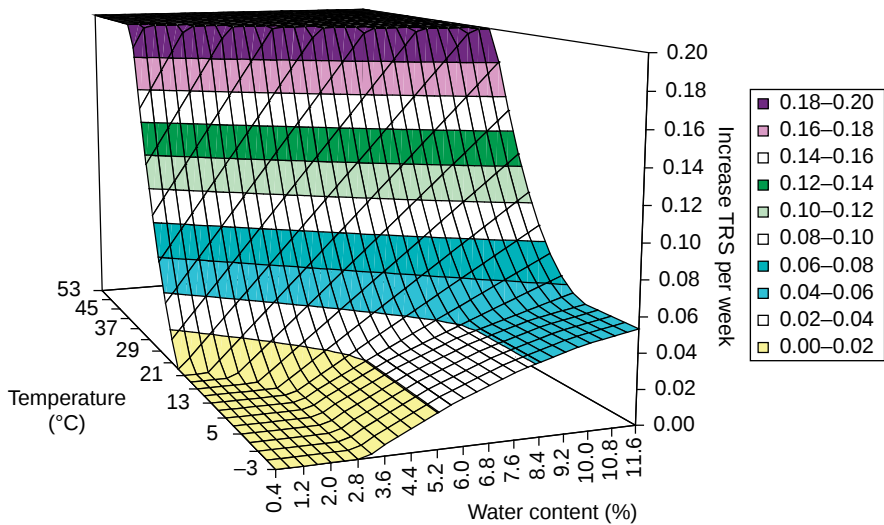


Figure 4 Response surface for amorphous form degradation.

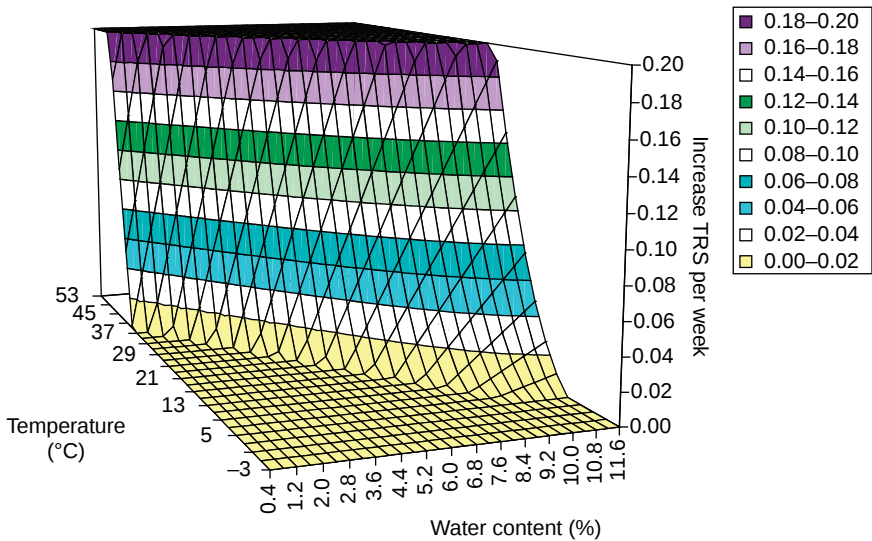


Figure 5 Response surface for crystalline form degradation.

To support the accuracy of the statistical model estimates of degradation rates, Arrhenius treatments of the stability results were also performed. An additional temperature (35°C) was used for each sample form to provide four points in the Arrhenius plots. Degradation rates at both high and low water content were used for the high and low temperature points. The intermediate water content was used at the intermediate temperatures. Arrhenius plots for peak B of the amorphous form and peak F of the crystalline form are shown in Figures 8 and 9, respectively. The effect of water content on degradation rate is apparent for peak B in the amorphous form by the spread of results for the two points at each of the high and low temperatures. A similar analysis was done for total impurities.

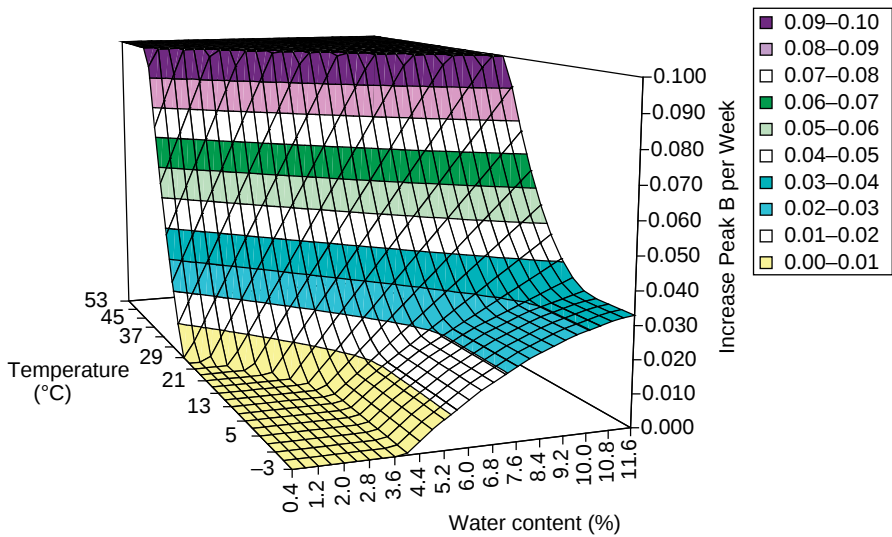


Figure 6 Response surface for peak B formation in amorphous form sample.

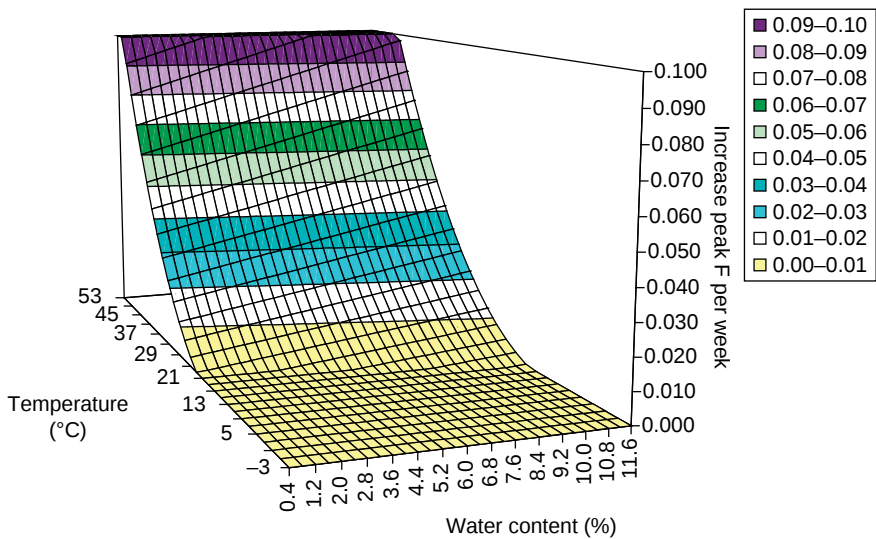


Figure 7 Response surface for peak F formation in crystalline form sample.

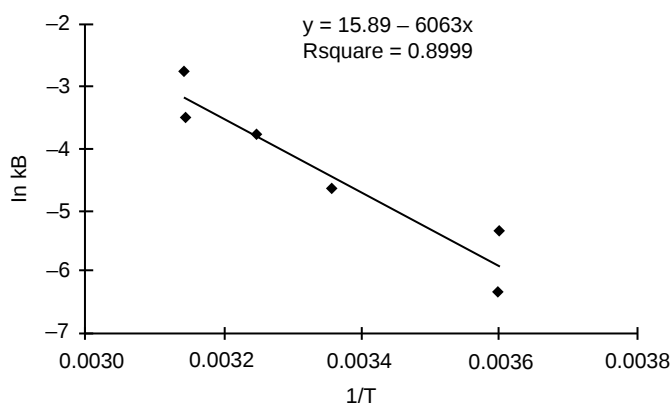


Figure 8 Arrhenius plot for peak B formation in amorphous form sample.

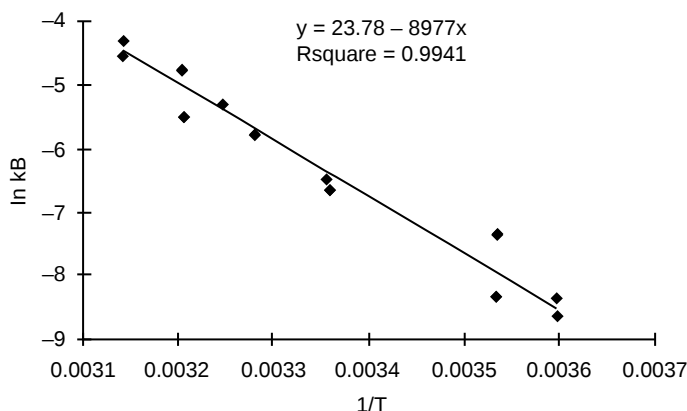


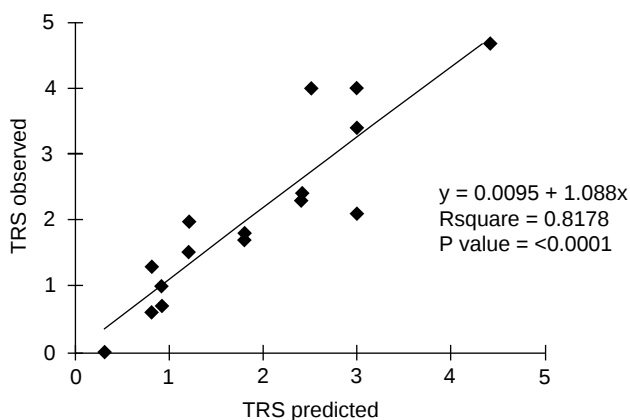
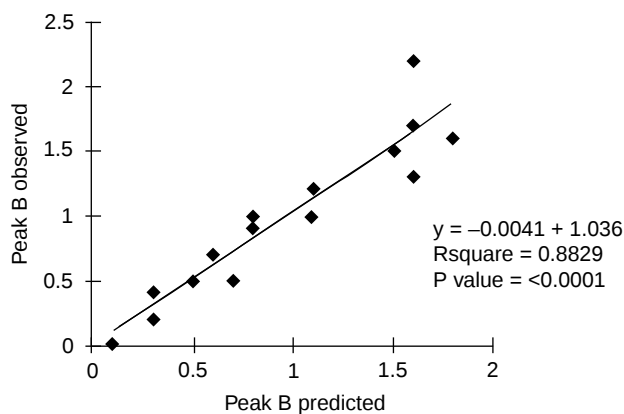
Figure 9 Arrhenius plot for peak F formation in crystalline form sample.

Predicted degradation rates using the statistical design study models and the Arrhenius data are compared to observed stability data in Table 2. Rates were calculated assuming an intermediate water content of about 6%. The agreement of predicted and actual results is very good. Good agreement was expected for the statistical model since the center point (25°C/6% water) was used to obtain the observed degradation rate. The 5°C data from the statistical design study were not used for the observed rates; however, since higher and lower water content samples were studied.

As a final check on the accuracy of the statistical model, the observed results for total related substances and peak B obtained during stability testing of 18 different manufacturing and lab-scale batches of drug substance were compared to predicted increases (Figs. 10 and 11). The stability studies represented amorphous and crystalline material with water content ranging from 3 to 8%. Temperatures used for stability studies were 5, 25, and 35°C. The duration of the studies was from 11 weeks to 10 months. The correlation of observed and predicted values was quite good. The intercepts were not significantly different from zero and the slopes were close to 1.0. The overall agreement and consistency of results leads to the conclusion that the estimates generated by the statistically designed models are reasonably good predictors of actual degradation rates under various conditions.

Table 2 Comparison of the Percent Degradation Products Formed Per Year Predicted from Statistically Designed Studies, Arrhenius studies and the Observed Rates

Product/ Temperature	Amorphous Form Arrhenius	Amorphous Form Stat. Model	Amorphous Form Observed	Crystalline Form Arrhenius	Crystalline Form Stat. Model	Crystalline Form Observed
Peak A, 25°C	4.2	3.8	3.1	0.90	1.0	1.3
Peak A, 5°C	1.0	0.8	1.0	0.04	<0.1	0.00
Peak B, 25°C	nd ^a	nd	nd	0.64	0.52	0.52
Peak B, 5°C	nd	nd	nd	0.07	<0.1	0.09
Peak C, 25°C	nd	nd	nd	0.12	0.10	0.09
Peak C, 5°C	nd	nd	nd	0.02	<0.1	0.00
TRS, 25°C	6.3	6.8	6.1	4.5	4.4	4.3
TRS, 5°C	0.9	1.3	1.1	0.58	<0.1	0.22

^aNot determined.**Figure 10** Comparison of results observed for total related substances from stability testing of different lots and the predicted values using statistically derived models.**Figure 11** Comparison of results observed for peak B from stability testing of different lots and the predicted values using the statistically derived models.

API INTERMEDIATE AND STARTING MATERIAL STABILITY ASSESSMENT

The terms “pharmaceutical stress testing” or “forced degradation” are most often applied to the study of active pharmaceutical ingredients (API). Stress testing provides useful insights into the inherent stability of a molecule and allows researchers to identify, measure, and control potential pathways of degradation. The value in characterizing and controlling API stability is immediately obvious; however, these merits are also valid for starting materials and synthetic API intermediates. Instability of these components may potentially lead to the manufacture of poor quality drug substance and will always result in lower API yields. In light of these considerations, it makes good quality and business sense to characterize and control the stability of starting materials and intermediates and to be able to monitor their stability with sensitive and selective analytical methods. Furthermore, a good understanding of starting material and intermediate stability fits well with a quality-by-design (QbD) approach to drug development and manufacturing.

It is critical to thoroughly understand the stability characteristics of drug substances and drug products and to establish controls to ensure that they are suitable for use throughout the designated shelf life. Because of the criticality of these activities, a great deal of emphasis is placed on these studies as is evidenced through the various guidance documents available that establish the framework for stability study design. In contrast, very little discussion or guidance exists for establishing a thorough understanding and control of starting material and intermediate stability. Yet, as the industry continues to expand global manufacturing networks and to embrace and apply QbD, the understanding of starting material and intermediate stability becomes even more important. For example, in a QbD new drug application (NDA) submission, it is conceivable that one may seek to bypass analytical testing of intermediates if a process is successfully executed within its validated design space. This could be an acceptable approach as any intermediate manufactured by a process executed within the bounds of a well-characterized design space will, by definition, forward process to make acceptable API. However, intermediate stability is typically not a factor considered when developing the process design space. Although external to the process design space, instability of an intermediate can have a significant impact on the acceptability of forward processed material even if the process is operated within the validated design space. Therefore, understanding the stability characteristics of starting materials and intermediates, including understanding stability differences among vendors and applicable synthetic routes, is of importance to developing an overarching control strategy.

At a high level, ICH Q1A(R2) provides guidance on the selection of temperature and humidity storage conditions for stress testing (3). This guidance states that stress conditions consist of temperatures, in 10°C increments above accelerated conditions (e.g., 50°C, 60°C, etc.) and relative humidity (RH) at or above that of accelerated conditions (e.g., ≥75% RH). Although this guidance is targeted toward API, these stress conditions are also suitable for starting material and intermediate testing. For example, based on an Arrhenius kinetic model using a conservative activation energy of 17 kcal/mol (71 kJ/mol), storage at 70°C/75% RH for 1 month is approximately equivalent to 43 months at room temperature (see chap. 19). In most cases, knowledge of the projected room temperature stability beyond 3.5 years is more than sufficient for a starting material. During a stress stability study, in most cases, multiple time points are collected over the duration of the experiment to allow one to gather experimental degradation rate data and to identify primary and secondary degradation products. In addition, to predicting the equivalent storage time at room temperature, one can use the Arrhenius kinetic model to easily project stability over shorter storage durations during the stress experiment. For example, if a material was stored at 70°C/75% RH and time points were collected every week, one could project room temperature stability for 10 months (1 week stress), 20 months (2 weeks stress), 30 months (3 weeks stress), and 40 months (4 weeks stress) for a 28 day stress study. Therefore, if a material did not show any degradation for 2 weeks stressed at 70°C/75% RH, with reasonable confidence it can be concluded that the material would not exhibit degradation for up to 20 months when held at 25°C/75% RH.

The amount of information that can be gathered quickly from this type of stress testing is immense. Not only do these experiments reveal the inherent solid-state stability of an intermediate or starting material, but they also provide data needed to support packaging (e.g., is protection from humidity needed?), initial re-evaluation dating of materials, and storage condition excursions. Because of the short amount of time and little overhead required to obtain this very useful dataset, it is recommended here to stress starting materials and intermediates, open dish, at 70°C/75% RH and 70°C/ambient RH conditions for 28 days collecting stability time points every 7 days. Note that different conditions and/or time points may be needed depending on the properties of the compound of interest.

The timing of when to conduct solid-state stress testing is case dependent and may vary based on project needs. However, it is suggested that stress testing be conducted at a minimum at (i) the time that the sequence of starting materials and intermediates is defined (i.e., selection of the molecule's synthetic route), (ii) when a different vendor of the starting material or intermediate is selected or being considered (see example below), and (iii) when the synthetic route, process, or scale at which the material is produced is altered. These minimum criteria are based on generating an understanding of potential complexities of developing a specific synthetic route due to unstable starting materials and intermediates, and to ensure that changes to synthetic route and/or process (by changing vendor, process or scale) does not affect the stability of the material.

STARTING MATERIALS FROM MULTIPLE SOURCES

Impurities in API starting materials are traditionally controlled through the use of specifications that are established based on the historical quality of starting materials received from vendors and related impurity rejection studies. Elevated temperature and humidity stress studies may be performed on representative material to characterize the inherent stability of the starting material. However, because of costs involved, it is uncommon to perform this activity on representative material from all sourced vendors or to repeat this activity if the vendor modifies their synthetic route, process, or scale of manufacture. Yet, this can be an informative endeavor as slight changes in a vendor's synthetic route or process can lead to unexpected material instability.

In the example below a starting material (Starting Material X) for the synthesis of a new chemical entity (NCE) was manufactured by three different vendors and was stressed to compare relative material stability and to identify appropriate packaging and environmental controls for storage. The stress testing was performed for 28 day at elevated temperature (60°C) and elevated temperature and humidity (60°C/75%RH). Samples were taken at approximately 7 day intervals and stored at subfreezing conditions prior to analysis. Samples were subsequently analyzed as a single batch.

Starting material vendors A and B utilized the same synthetic route (SR-1) but executed slightly different processes, while vendor C used a different synthetic route (SR-2) and process. As shown by the chromatograms in Figure 12 and the analytical data in Table 3, vendor C has the lowest impurity content prior to stressing. Potency and chiral purity results for all vendors were within analytical method variability. All three vendor samples demonstrated excellent stability at 60°C (open dish; ambient humidity) throughout the entire study; no degradation was observed. At 60°C/75% RH (open dish) for 7 days both vendors A and B exhibited an insignificant amount of degradation. However, vendor C demonstrated significant degradation with the formation of nine degradation products and an increase in total impurities of greater than five times the original unstressed level (Table 4, Fig. 13). At further time points (Table 5, Fig. 14), vendors A and B materials formed a single degradation product whereas material from vendor C formed 11 different degradation products and eventually deliquesced. As part of stability-indicating method development, stress testing of this starting material in solution was performed across a broad pH range to model hydrolytic degradation, and via radical initiators and hydrogen peroxide to investigate oxidative pathways (see section "Rapid

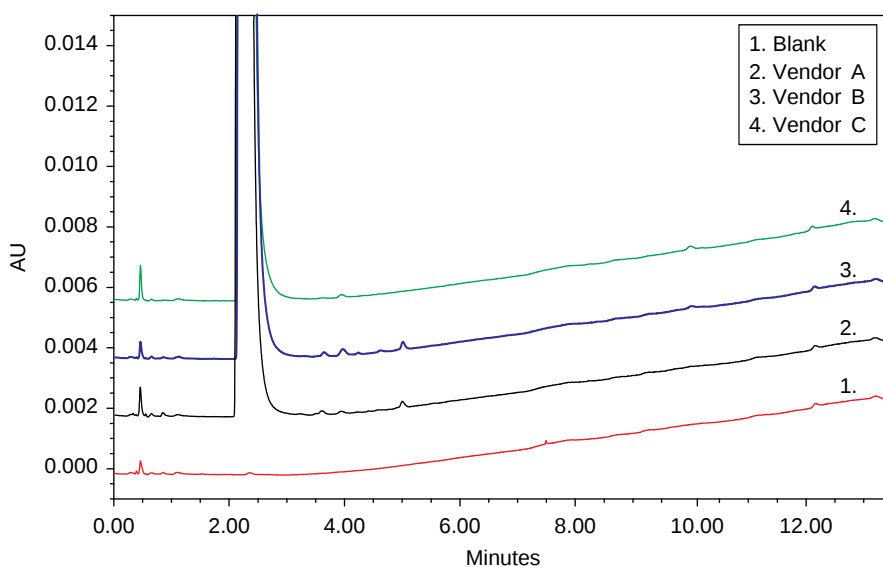
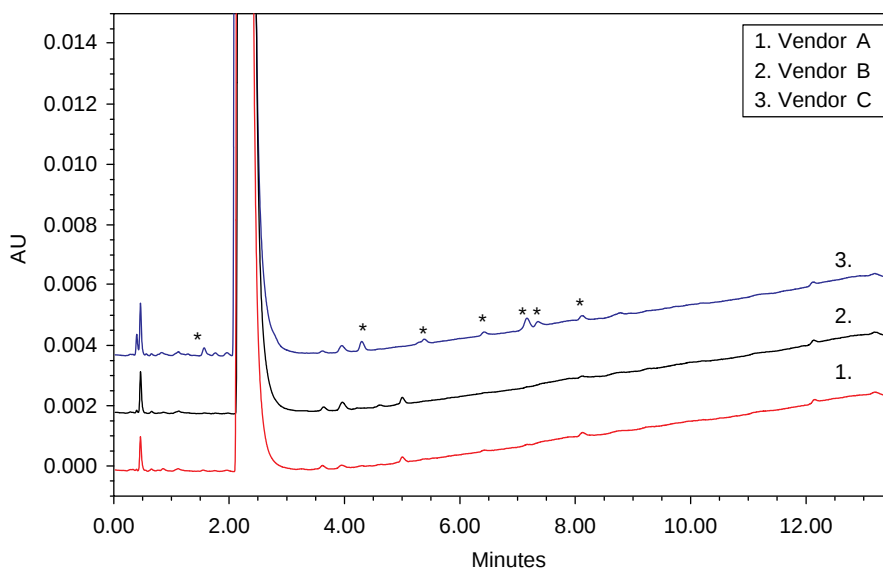


Figure 12 Chromatograms of unstressed Starting Material X.

Table 3 Analytical Results for Starting Material X

Vendor	Synthetic Route	Potency (%)	% Total Impurities	% Largest Impurity	% Chiral Purity
Vendor A	SR-1	100.1	0.13	0.07	100.0
Vendor B	SR-1	99.8	0.21	0.08	99.4
Vendor C	SR-2	99.1	0.09	0.05	99.9

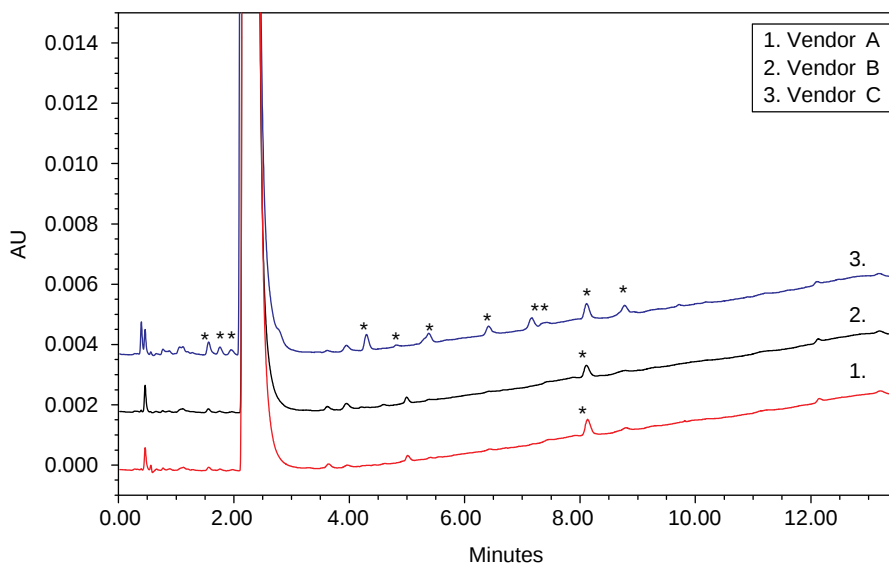


*Denotes degradation product

Figure 13 Chromatograms of Starting Material X after 7 days at 60°C/75% RH.

Table 4 Analytical Results for Starting Material X After 7 Days at 60°C/75% RH

Vendor	Synthetic Route	No. of Degradation Products Formed	% Largest Degradation Product (>0.05%)	% Total Impurities	% Largest Impurity
Vendor A	SR-1	0	0.0	0.21	0.06
Vendor B	SR-1	0	0.0	0.21	0.09
Vendor C	SR-2	9	0.11	0.48	0.11



*Denotes degradation product

Figure 14 Chromatograms of Starting Material X after 28 days at 60°C/75% RH.**Table 5** Analytical Results for Starting Material X After 28 Days at 60°C/75% RH

Vendor	Synthetic Route	No. of Degradation Products Formed	% Largest Degradation Product	% Total Impurities	% Largest Impurity
Vendor A	SR-1	1	0.19	0.35	0.19
Vendor B	SR-1	1	0.16	0.37	0.16
Vendor C	SR-2	11	0.13	0.96	0.13

Assessment of Stability-Indicating Method Capability” for further discussion). The degradation observed from solid-state stress testing appears to be primarily the result of an oxidative degradation pathway based on chromatographic comparison.

The two most likely causes that would lead to variations in stability as described in the previous paragraph could be either differences in material crystallinity or low-level impurities that could lead to decreased material stability (52,53). Materials manufactured using a different process can lead to the formation of different crystal forms, salts, or non-crystalline amorphous material. Different impurity profiles may also result. In this example, x-ray powder

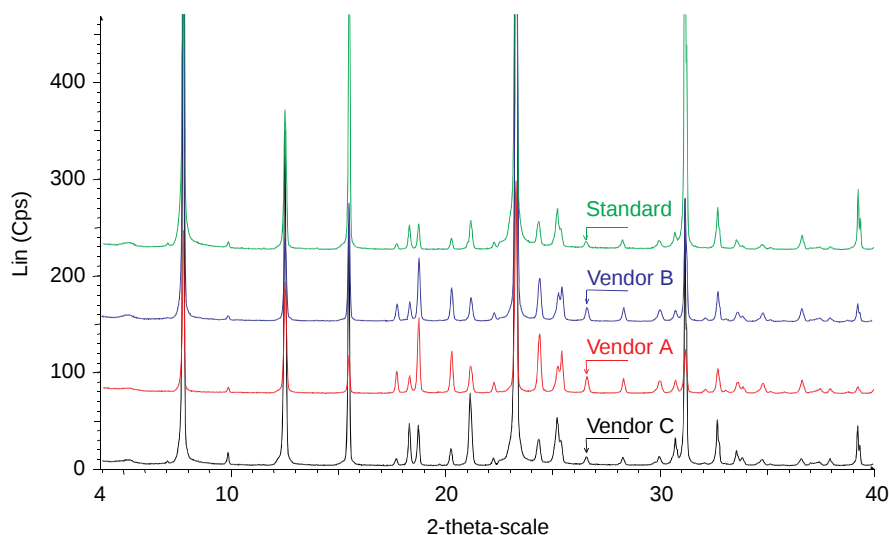


Figure 15 Crystallinity: XRPD of vendor samples of Starting Material X.

diffractograms were collected for each vendor sample for comparison to each other and to a well-characterized reference standard (Fig. 15). The well-defined peaks in diffractograms and the similar baseline offset indicates highly crystalline materials of the same crystal form for the three vendor samples. However, the possibility of differences in low levels of amorphous material (e.g., <5%) could not be ruled out due to the insensitivity of XRPD for detection of amorphous content. Therefore, we did not definitively determine the source of the differences in stability. Nevertheless, this example highlights how very slight, and sometimes unknown, differences between materials can lead to variable stability.

Controlling the quality of starting materials used in the synthesis of an API is critical to the final quality of the API. Optimization efforts early in process development may lead to an alternate salt or solvate, different polymorph (or amorphous) or the presence of a new low-level impurity. Rapid stress screening can be used to provide quick insight to the potential impact of these potential changes on material stability.

RAPID ASSESSMENT OF STABILITY-INDICATING METHOD CAPABILITY

In order to evaluate the stability of starting materials and intermediates, stability-indicating analytical methods must be available for control strategy development and subsequent stability monitoring. Stressed samples provide the foundation upon which stability-indicating methods can be developed and assessed. Oxidative, hydrolytic, and photo stressing of material in solution can quickly generate such samples for use in method development. Often times when solution stress testing is applied for the understanding of the inherent stability of an API, a broad range of conditions (e.g., a wide pH range) is explored and many time points are collected as to enable the assessment of degradation kinetics. However, detailed solution stress-testing studies are not necessary for generating samples to be used in stability-indicating method development. Abbreviated API stress-testing protocols can provide samples for method development quickly, although there are compromises that are necessary. For example, if multiple time points are not collected during the degradation study, one does not have as clear a picture of the degradation profile and could be misdirected by developing a method around secondary or higher-order degradation products. However, in light of these potential

issues for analytical method development, an abbreviated solution stress-testing approach is advantageous in that it permits rapid execution of experiments, generates a limited number of data sets that require analysis, generates information regarding inherent compound stability, and generates materials useful for method development and/or characterization.

A solution stress-testing protocol that can be executed within a few hours and have materials ready for analysis or method development by the next day is desirable. The development of a simple, streamlined protocol that minimizes powder weighing operations and uses a single sample stock solution, a single sample solvent for all experiments, and a single (24 hour) time point, can achieve this goal. An example protocol that follows this outline is shown in Table 6. Additional details that should be considered when developing a solution stress-testing protocol are mentioned below.

Table 6 Example 24 hours' Solution Stress-Testing Protocol for Starting Materials and Intermediates for Stability Indicating Method Development or Assessment

Test	Sample Preparation
	Starting material/intermediate stock solution prepared at ~2.0 mg/mL
Photostability	<i>Sample preparation:</i> Add 5 mL of stock solution to a 10 mL volumetric flask and dilute to volume with diluent. Cap flask and place in a photostability chamber for 240,000 lux-hour exposure [24 hours in a photostability chamber at 10,000 lux light intensity; simulates 10 days of constant light exposure on a well-lit (1000 lux light intensity) laboratory bench top] <i>Control preparation:</i> Repeat the sample preparation step and subsequently wrap the flask in aluminum foil and place in the photostability chamber with the sample.
Nucleophilic oxidation	<i>Sample preparation:</i> Add 5 mL of stock solution to a 10 mL volumetric flask. Add 1 mL of 3% hydrogen peroxide, immediately dilute to volume with diluent, cap and shake well (do not heat). Store samples in a dark place. <i>Control preparation:</i> Add 9 mL of diluent to a 10 mL volumetric flask. Add 1 mL of 3% hydrogen peroxide, cap and shake well. Store samples in a dark place.
Radical-initiated oxidation	<i>AIBN or ACVA stock preparation (1 mg/mL):</i> Prepare a 1 mg/mL solution of AIBN or ACVA (selection based on solubility ACVA—water soluble/AIBN—organic soluble). <i>Sample preparation:</i> Add 5 mL of starting material/intermediate stock solution to a 25 mL volumetric flask. Add 5 mL of AIBN/ACVA stock solution and mix well. Cap flask and place in a 40°C oven. <i>AIBN/ACVA control preparation:</i> Add 5 mL of diluent to a 25 mL volumetric flask. Add 5 mL of AIBN/ACVA stock solution and mix well. Cap flask and place in a 40°C oven. <i>Thermal control preparation:</i> Add 5 mL of starting material/intermediate stock solution to a 25 mL volumetric flask. Add 5 mL of diluent and mix well. Cap flask and place in a 40°C oven. <i>Note:</i> Do NOT dilute samples to volume. It is imperative that the volume of stress sample only consume ~50% or less of the total volume of the capped container (54).
Hydrolysis	<i>Acidic and basic sample preparation:</i> Add 4 mL of diluent and 5 mL of stock solution to a scintillation vial. Add 1 mL of 1.0 N HCl or 1.0 N NaOH to the scintillation vial and mix well to make a 0.1 N HCl solution. Place in a 40°C oven. <i>Control preparation:</i> Add 1 mL of 1.0 N HCl or 1.0 N NaOH and 9 mL of diluent to a scintillation vial. Place in a 40°C oven.
Transition metals	<i>Iron(III) chloride hexahydrate stock preparation (2 mM):</i> Weigh approximately 13.6 mg into a 25 mL volumetric flask and dilute to volume with diluent. <i>Copper(II) chloride stock preparation (2 mM):</i> Weigh approximately 6.8 mg into a 25 mL volumetric flask and dilute to volume with diluent. <i>Sample preparation:</i> Add 5 mL of starting material/intermediate stock solution to a 25 mL volumetric flask. Add 5 mL of copper II chloride or iron III chloridehexahydrate stock solution and mix well. Cap flask and place in a 40°C oven. <i>Metals control preparation:</i> Add 5 mL of diluent to a 25 mL volumetric flask. Add 5 mL of copper chloride or iron chloridehexahydrate stock solution and mix well. Cap flask and place in a 40°C oven.

Sample solvent considerations: A mixed solvent consisting of an organic and aqueous component is recommended to provide adequate dissolution of the material being tested and potential degradation products that are formed. It is recommended to use a sample diluent comprised of an organic/aqueous ratio similar to that of the diluent used in the intended analytical method. If the analytical method sample diluent consists of either all aqueous or all organic, the addition of at least 5% of the un-utilized phase is recommended. Methanol is recommended as a sample solvent component for oxidative susceptibility testing since it acts as a scavenger for alkoxyl radicals (which are extremely aggressive oxidants and give rise to unrealistic oxidative degradation products) formed during the radical initiator test, but does not interfere with propagation of the desired peroxy radical (see chap. 6 for more discussion of this topic).

Solution photostability stressing: Photostability testing of intermediates and starting materials in solution is useful for identifying potential photostability issues that may occur during material handling. The vast majority of photodegradation is likely to be during analytical handling and testing while the material is in solution (e.g., exposure of analytical solutions to laboratory lighting prior to and during analysis). Therefore, to best simulate the common fluorescent laboratory lighting, the recommended photostability testing conditions consist of a chamber fitted with cool white fluorescent lamps similar to those used in the laboratory where the analytical work will take place.

Oxidation (nucleophilic and radical-initiated): Oxidation is one of the most common pharmaceutical degradation pathways. There are three main oxidative degradation pathways: (i) nucleophilic oxidation, (ii) radical-initiated oxidation (autoxidation), and (iii) transition metal-induced oxidation. Since radical-initiated and nucleophilic oxidation are the most commonly observed oxidation pathways, attempting to degrade the starting materials and intermediates by these pathways is recommended.

Nucleophilic oxidation: Nucleophilic oxidation of secondary and tertiary amines and thioether (R-S-R) groups by trace levels of hydroperoxides to form *N*-oxides and sulfoxides, respectively, is common. The potential formation of these degradation products can be explored by exposure to hydrogen peroxide (0.3% hydrogen peroxide in sample solvent is sufficient). The stress solution should remain unbuffered, as protonating amine groups using low-pH buffered solutions will inhibit this degradation mechanism yielding artificially stable results. Solutions should not be heated as heating leads to the formation of hydroxyl radicals (HO•) that can lead to an unrealistic degradation profile. In addition, stress samples should be stored capped and protected from light to reduce the potential for photo-induced hydroxyl radical production.

Radical-initiated oxidation: Pharmaceutical oxidative degradation is often the result of autoxidation (radical-initiated). Radical initiators, such as 2,2'-azobisisobutyronitrile (AIBN) and 4,4'-azobis-4-cyanovaleric acid (ACVA), have been shown to be good predictors of natural autoxidative processes. For further discussion of oxidative degradation mechanisms and stress testing, see chapter 6.

Hydrolysis: Acid and base hydrolysis is a very common pharmaceutical degradation pathway, particularly for amide-, imide-, and ester-containing species. To assess the propensity for a starting material/intermediate to degrade via this pathway, dissolution and storage of the material in 0.1 N HCl and 0.1 N NaOH is recommended. It should be noted here that in order to achieve a rapid protocol using a single sample solvent, it is possible to end up performing hydrolysis studies in a methanol containing diluent. This may result in acid-catalyzed esterification which may result in degradation artifacts.

Transition metals: Trace levels of iron, copper, or other metals in reagent water, from manufacturing equipment, leaching from glassware, and other potential sources, can result in metal catalyzed oxidation of drug-related molecules. To assess the susceptibility of a starting material/intermediate to degrade via this pathway, dissolution and storage of the material in an unbuffered solution of 1 mM copper II or iron III chloride at RT to 40°C is recommended.

SUMMARY AND CONCLUSIONS

Comparative stability studies done using stress conditions can be valuable in providing a rapid indication of the relative stability properties of the samples being compared. Appropriate information concerning the applicability of stress results to room temperature is necessary to draw meaningful conclusions from the stress studies. The amount of this information needed depends on the degree of confidence the investigator requires and how the results will be used. Simple studies such as direct comparisons or more complex statistical design studies can provide valuable information regarding the effect of manufacturing changes, storage conditions, or sample characteristics on stability. This information can help direct further development efforts or justify processing changes appropriately. Comparative stress stability studies are also useful for evaluating synthetic starting materials or intermediates which may be obtained from multiple suppliers and possibly from different manufacturing processes. A rapid stress protocol was shown to be useful in the development of analytical methods and confirmation of the stability-indicating capability of a method. This confirmation supports the validity of the analytical methods that are the underpinning for all stability evaluations.

REFERENCES

1. Carstensen JT. In: Grimm W, Schepky G, eds. *Stabilitätsprüfung in der Pharmazie*. Aulendorf: Editio Cantor, 1980: 11.
2. Grimm W. General concept for stability testing. In: Grimm W, Krummen K, eds. *Stability Testing in the EC, Japan, and the USA*. Stuttgart: Wissenschaftliche Verlagsgesellschaft, 1993: 191–223.
3. International Conference on Harmonisation. *Stability Testing of New Drug Substances and Products Q1A(R2)* Fed Register 2003; 68: 65717–18.
4. FDA guidelines for Scale-up and Postapproval Changes (SUPAC) and changes to an approved NDA or ANDA.
5. Lin SL. Parenteral formulations: I. Comparison of accelerated stability data with shelf-life studies. *Bull Parenter Drug Assoc* 1969; 23: 269–88.
6. Pope DG. Accelerated stability testing for prediction of drug product stability. *Drug Cosmet Ind* 1980; 127(5): 54, 56, 59, 60, 62, 116.
7. Pope DG. Accelerated stability testing for prediction of drug product stability. *Drug Cosmet Ind* 1980; 127(6): 48, 50, 55–56, 58, 60, 62, 64–6.
8. Yang W, Roy SB. Projection of tentative expiry date from one-point accelerated stability testing. *Drug Dev Ind Pharm* 1980; 6: 591–604.
9. Kowalski K, Beno M, Bergstrom C, Gaud H. The application of multi-response estimation to drug stability studies. *Drug Dev Ind Pharm* 1987; 13: 2823–38.
10. Young WR. Accelerated temperature pharmaceutical product stability determinations. *Drug Dev Ind Pharm* 1990; 16: 551–69.
11. Yoshioka S, Aso Y, Izutsu K, Terao T. Application of accelerated testing to shelf-life prediction of commercial protein preparations. *J Pharm Sci* 1994; 83: 454–6.
12. Yoshioka S, Aso Y, Takeda Y. Statistical evaluation of accelerated stability data obtained at a single temperature: I. Effect of experimental errors in evaluation of stability data obtained. *Chem Pharm Bull* 1990; 38: 1757–9.
13. Yoshioka S, Aso Y, Takeda Y. Statistical evaluation of accelerated stability data obtained at a single temperature: II. Estimation of shelf-life from remaining drug content. *Chem Pharm Bull* 1990; 38: 1760–2.
14. Gneuss KD. Prediction of the stability of drug products, new techniques and strategies. In: Grimm W, Krummen K, eds. *Stability Testing in the EC, Japan, and the USA*. Stuttgart: Wissenschaftliche Verlagsgesellschaft, 1993: 75–94.
15. Stamper GF, Lambert WJ. Accelerated stability testing of proteins and peptides: pH-stability profile of insulinotropin using traditional Arrhenius and nonlinear fitting analysis. *Drug Dev Ind Pharm* 1995; 21: 1503–11.
16. Waterman KC, Adami RC. Accelerated aging: Prediction of chemical stability of pharmaceuticals. *Int J Pharm* 2005; 239: 101–25.
17. Waterman KC, Carella AJ, Gumkowski MJ, Lukulay P, MacDonald BC, Roy MC, Shamblin SL. Improved protocol and data analysis for accelerated shelf-life estimation of solid dosage forms. *Pharm Res* 2007; 24: 780–90.

18. Chafetz L. Practical testing of solid-state stability of pharmaceuticals. *J Pharm Sci* 1992; 81: 107.
19. Yang W. Errors in the estimation of the activation energy and the projected shelf life in employing an incorrect kinetic order in an accelerated stability test. *Drug Dev Ind Pharm* 1981; 7: 717–38.
20. Baertschi SW. Forced degradation and its relationship to real time drug product stability. In: *Proceedings from the AAPS Stability Workshop held in September 2007, Bethesda, MD*, Huyn-Ba, K., ed. New York: Springer, 2009.
21. Cleland JL, Powell MF, Shire SJ. The development of stable protein formulations: a close look at protein aggregation, deamidation, and oxidation. *Crit Rev Ther Drug Carrier Syst* 1993; 10: 307–77.
22. Gu LC, Erdos EA, Chiang HS, Calderwood T, Isai K, Visor GC, Duffy J, Hsu WC, Foster LC. Stability of interleukin 1b in aqueous solution: analytical methods, kinetics, products, and solution formulation implications. *Pharm Res* 1991; 8: 485–90.
23. Franks F. Accelerated stability testing of bioproducts: attractions and pitfalls. *Trends Biotechnol* 1994; 12: 114–17.
24. S. Görög. The sacred cow: the questionable role of assay methods in characterising the quality of bulk pharmaceuticals. *J Pharm Biomed Anal* 2005; 36: 931–7.
25. Hofer JD, Olsen BA, Rickard EC. Is HPLC assay for drug substance a useful quality control attribute? *J Pharm Biomed Anal* 2007; 44: 906–13.
26. Skrdla PJ, Wang T, Antonucci V, et al. Use of a quality-by-design approach to justify removal of the HPLC weight % assay from routine API stability testing protocols. *J Pharm Biomed Anal* 2009; 50: 794–6.
27. Koenigbauer MJ. Pharmaceutical applications of microcalorimetry. *Pharm Res* 1994; 11: 777–83.
28. Buckton G, Gaisford S. The use of microcalorimetry in stress testing. In: Baertschi SW, ed. *Pharmaceutical Stress Testing*. Boca Raton, FL: Taylor & Francis, 2005: 327–54.
29. Duddu S, Weller K. Importance of glass transition temperature in accelerated stability testing of amorphous solids: case study using a lyophilized aspirin formulation. *J Pharm Sci* 1996; 85: 345–7.
30. Ahlneck C, Zograf G. The molecular basis of moisture effects on the physical and chemical stability of drugs in the solid state. *Int J Pharm* 1990; 62: 87–95.
31. Byrn S, Pfeiffer RR, Stowell JG. *Solid State Chemistry of Drugs*. West Lafayette: SSCI, Inc., 1999: 443–7.
32. Goldberg R, Nightingale CH. Stability of aspirin in propoxyphene compound dosage forms. *Am J Hosp Pharm* 1977; 34: 267–69.
33. Furlanetto S, Mura P, Gratteri P, Pinzauti S. Stability prediction of cefazolin sodium and cephaloridine in solid state. *Drug Dev Ind Pharm* 1994; 20: 2299–313.
34. Barthomeuf C, Pourrat H, Pourrat A, Ibrahim H, Cottier PE. Stabilization of octastatin, a somatostatin analogue: comparative accelerated stability studies of two formulations for freeze-dried products. *Pharmaceutica Acta Helvetiae* 1996; 71: 161–6.
35. Felton LA, Yang J, Shah K, Omidian H, Rocca JG. A rapid technique to evaluate the oxidative stability of a model drug. *Drug Dev Ind Pharm* 2007; 33: 683–9.
36. Black JC, Layloff T. Summer of 1995—Mailbox temperature excursions in St. Louis. *Pharm Forum* 1996; 22: 3305.
37. Carstensen JT, Rhodes CT. Cyclic temperature stress testing of pharmaceuticals. *Drug Dev Ind Pharm* 1993; 19: 401–3.
38. Bempong DK, Mirza T, Grady LT, Lindauer RF. Accelerated screening studies to identify labile preparations (1). *Pharm Forum* 1999; 25: 8929–38.
39. Bempong DK, Mirza T, Grady LT, Lindauer RF. Accelerated screening studies to identify labile preparations (2). *Pharm Forum* 1999; 25: 8939–46.
40. Bempong DK, Mirza T, Bradby S, Grady LT, Lindauer RF. Open-dish study to identify labile preparations (1). *Pharm Forum* 1999; 25: 8947–55.
41. Adkins RE, Mirza T, Bempong DK, Grady LT, Lindauer RF. Open-dish study to identify labile preparations (2). *Pharm Forum* 1999; 25: 8956–63.
42. Waterman KC. Understanding and predicting pharmaceutical product shelf-life. In: Huyn-ba K, ed. *Handbook of Stability Testing in Pharmaceutical Development*. New York: Springer, 2009.
43. Naversnik K, Bohanec S. Predicting drug hydrolysis based on moisture uptake in various packaging designs. *Eur J Pharm Sci* 2008; 35: 447–56.
44. Olsen BA, Perry FM, Snorek SV, Lewellen PL. Accelerated conditions for stability assessment of bulk and formulated cefaclor monohydrate. *Pharm Dev Tech* 1997; 2: 303–12.
45. Lorenz LJ, Bashore FN, Olsen BA. Determination of process-related impurities and degradation products in cefaclor by high-performance liquid chromatography. *J Chrom Sci* 1992; 30: 211–16.

46. Dorman DE, Lorenz LJ, Occolowitz JL, et al. Isolation and structure elucidation of the major degradation products of cefaclor in the solid state. *J Pharm Sci* 1997; 86: 540–9.
47. Box GEP, Hunter WG, Hunter JS. *Statistics for Experimenters*. New York: Wiley, 1978.
48. Jones SP. Stability and response surface methodology. In: Hendriks MMWB, DeBoer JH, Smilde AK, eds. *Robustness of Analytical Chemical Methods and Pharmaceutical Technological Products*. Amsterdam: Elsevier, 1996: 11–77.
49. Remunan C, Bretal MJ, Nunez A, Jato JLV. Accelerated stability study of sustained-release nifedipine tablets prepared with Gelucire. *Int J Pharm* 1992; 80: 151–9.
50. Nguyen NAT, Wells ML, Cooper DC. Identification of factors affecting preservative efficacy and chemical stability of lamivudine oral solution through statistical experimental design. *Drug Dev Ind Pharm* 1995; 21: 1671–82.
51. Bos CE, Bolhuis GK, Smilde AK, DeBoer JH. The use of a factorial design to evaluate the physical stability of tablets after storage under topical conditions. In: Hendriks MMWB, DeBoer JH, Smilde AK, eds. *Robustness of Analytical Chemical Methods and Pharmaceutical Technological Products*. Amsterdam: Elsevier, 1996: 309–41.
52. Guerrieri P, Salameh AK, Taylor LS. Effect of small levels of impurities on the water vapor sorption behavior of ranitidine HCl. *Pharm Res* 2007; 24: 147–56.
53. Guerrieri PP, Smith DT, Taylor LS. Phase behavior of ranitidine HCl in the presence of degradants and atmospheric moisture-impact on chemical stability. *Langmuir* 2008; 24: 3850–6.
54. Nelson ED, Harmon PA, Szymanik RC et al. Evaluation of solution oxygenation requirements for azonitrile based oxidative forced degradation studies of pharmaceutical compounds. *J Pharm Sci* 2006; 95: 1527–39.

19 | Stress testing as a predictive tool for the assessment of potential genotoxic degradants

Steven W. Baertschi, David DeAntonis, Alan P. McKeown, Joel Bercu, Stephen Raillard, and Christopher M. Riley

INTRODUCTION

Strategies for dealing with genotoxic impurities (GTIs) or potential genotoxic impurities (PGIs) arising from drug synthesis have received considerable attention in the literature (1–6). In contrast to process impurities, genotoxic degradants have received less attention but may require different considerations since there is no opportunity for purification and their presence needs to be considered over the entire shelf-life of the product. This chapter outlines the key considerations that may be involved in understanding the risk to a drug development program of discovering that a degradant may have the potential for genotoxicity.

Strategies are needed not only to analyze for potentially genotoxic degradants, but also to understand their rate of production over the shelf-life of the product under the intended storage conditions. The determination of potentially GTIs at very low levels (sometimes sub-ppm) in the drug product is particularly challenging due to the presence of other impurities arising not only from the drug itself but also from excipients, all of which may interfere with the analysis. Stressing the drug product with heat, light, acid, base, or oxidants further complicates the analytical challenges due to the potential for the production of additional degradants of the drug substance, its reaction with excipients or with other impurities.

The use of stress testing in combination with other studies offers the possibility of predicting the most likely pathways of API degradation and the rate at which potentially genotoxic degradants are formed. In turn, these studies may help us to develop formulation strategies that reduce the risk of producing potentially genotoxic degradants at levels that need to be controlled in the drug product (Tables 1 and 2).

Toxicology and Regulatory Guidelines

The concern for impurities and degradants that are genotoxic is that they can cause damage to the DNA, resulting in a carcinogenic risk. While rodent and/or human carcinogenicity information is preferred to evaluate the cancer risk of a compound, for practical reasons this information is typically not available for a potential impurity/degradant and cancer risks are assessed based on limited data. In December 2002, the Committee for Medicinal Products for Human (CMP) of the European Medicines Agency (EMA) published a draft “Guideline on the Limits of GTIs.” The final document was published in June 2006 and became officially effective in January 2007 (1). The objectives of this European Guideline were to provide additional guidance for the control of GTIs in pharmaceuticals as an adjunct to the ICH Guidelines on Impurities in the drug substance [Q3A (R2)] and drug product [Q3B (R2)], and to provide more consistency in the review process within the EMA states. In December 2008, The FDA published its draft guidance for industry (3) “Genotoxic and carcinogenic impurities in drug substances and drug products: Recommended approaches.” That document (3) is not discussed here because at the time of writing of this chapter it was in the public comment phase and changes were anticipated.

When assessing the cancer risks of impurities and degradants, the EMA differentiated genotoxic compounds with a threshold dose–response relationship from those with a nonthreshold dose–response relationship. In the absence of sufficient experimental data to the contrary, it is assumed that genotoxic compounds (especially those that are mutagenic in the Ames assay) have the potential to directly damage DNA at any level of exposure and these

Table 1 Comparison of Qualification Thresholds (Maximum Allowable Intake) in µg/day for Genotoxic and Carcinogenic Degradants Proposed EMEA, FDA and Müller et al. (4)

Source	Maximum Daily Intake and Duration of Exposure (µg/day)						
	Single Dose	<14 days	14 days to 1 mo	1 to 3 mo	3 to 6 mo	6 to 12 mo	>12 mo
EMA (2)	120	–	60	20	10	5	–
Müller et al. (4)	–	–	120	40	20	10	1.5
FDA (draft) (3)	–	120	60	20	10	5	1.5

Table 2 Comparison of ICH Identification Thresholds for Degradants in Drug Substance (DS) and Drug Product (DP) and the Concentration of Degradants Calculated from the Proposed FDA Staged TTC Values for Various Daily Doses of Drug

Daily Dose (mg)	ICH Identification Thresholds (%)	Maximum Concentration of Genotoxic Degradants Calculated from the Proposed FDA Staged-TTC Values (%) ^a						
		DS	DP	<14 days (MDI: 120 µg)	14 days to 1 mo (MDI: 60 µg)	1–3 mo (MDI 20 µg)	3–6 mo (MDI: 10 µg)	6–12 mo (5 µg)
3000	0.05	0.10	0.0040	0.002	0.000,67	0.000,33	0.000,17	0.000,05
1000	0.10	0.2	0.012	0.0060	0.0020	0.000,10	0.000,50	0.000,15
300	0.10	0.2	0.040	0.020	0.0067	0.0033	0.0017	0.00050
100	0.10	0.2	0.12	0.060	0.020	0.010	0.0050	0.0015
30	0.10	0.2	0.40	0.20	0.067	0.033	0.017	0.0050
10	0.10	0.2	1.2	0.60	0.20	0.10	0.05	0.015
3	0.10	0.5	4.0	2.0	0.67	0.33	0.17	0.050
1	0.10	0.5	12	6.0	2.0	1.0	0.50	0.15
0.3	0.10	1.0	40	20	6.7	3.3	1.7	0.50

The numbers in bold indicate values that equal or exceed the ICH Identification Threshold for degradants in the drug substance. The underlined numbers equal or exceed ICH identification threshold for degradants in both the drug substance and the drug product.

compounds are categorized as nonthresholded. For carcinogens with a nonthreshold-related mechanism, an acceptable risk can be calculated based on its carcinogenic potency. The accepted excess risk for genotoxic carcinogens listed the ICH guideline on residual solvents [Q3C (R)] is 1 in 100,000 (10^{-5}).

When the carcinogenic potency of a GTI is unknown, the EMEA guideline introduced the concept of the threshold of toxicological concern (TTC) as an acceptable exposure. The TTC concept is based on the threshold of regulation previously adopted by the FDA for extractables and leachables in food-contacting material (7), and was derived from analysis of a database of 343 known carcinogens. This database was later expanded to more than 700 carcinogens and resulted in a threshold of regulation of 0.15 µg/day, corresponding to a lifetime increased risk of cancer of 1 in 10^6 . The proposed TTC of 1.5 µg/day for genotoxic or potentially genotoxic impurities (GTIs or PGIs) in pharmaceuticals corresponds to an increased cancer risk of 1 in 10^5 , recognizing the potential for therapeutic benefit to patients.

For compounds where experimental data exist to demonstrate that levels must be above a certain threshold before a genotoxic effect is observed, a permissible daily exposure (PDE) can be determined using the methods outlined in ICH guideline on residual solvents [Q3C (R)]. Calculation of the PDE involves applying safety factors to the no observed effect level (NOEL) or lowest observed effect level (LOEL) from the most relevant *in vivo* study. There are number of mechanisms of genotoxicity, which would be expected to have a threshold, including aneugenicity, topoisomerase inhibition, and inhibitors of DNA synthesis (1).

The EMEA guideline states that “higher limits may be justified under certain conditions such as short-term exposure periods” but does not go into specifics. Furthermore, the EMEA guideline was intended to apply to marketing authorization applications and did not address short-term exposures in clinical trials. The absence of specific guidance for clinical trials led to much debate in the pharmaceutical community during the period 2002–2006 and ultimately resulted in a proposal by an industry group (drawn from 12 companies) for the application of the staged-TTC (s-TTC) approach (4) (Table 1) in which the maximum daily exposure of a known genotoxic or potentially genotoxic impurity decreases with an increase in the length of treatment. A more conservative increased cancer risk of 1 in 10^6 was used in the s-TTC approach when calculating the maximum daily intake (MDI) values (Table 1) to accommodate the common inclusion of healthy volunteers in clinical trials for whom no therapeutic benefit would be derived. The concept of the s-TTC approach in clinical development was accepted by the EMEA in a question and answer document (2) published in June 2008 with reduced allowable daily intake values (Table 1) compared with those proposed by Müller et al. (4), incorporating a dose rate correction factor of 2 to account for deviations from the linear model extrapolation.

In most cases, with either the TTC or s-TTC approach, allowable limits for a genotoxic degradant will be lower than the impurity limits allowed by the ICH guidances [Q3A and B, (R2)] (Table 2). Therefore, it is important to develop scientific approaches toward understanding the risk of forming a genotoxic degradant.

RISK ASSESSMENT

Any degradant that forms at levels above ICH identification thresholds under relevant storage conditions over the shelf-life should undergo an assessment for genotoxic potential, involving structural elucidation followed by interrogation of the structure for genotoxic alerts, *in-cerebro* and *in-silico*, using computer programs such as Toxtree™, DEREK™, or MultiCASE™. One of the most challenging aspects of this topic is that degradants with the potential for genotoxicity may have to be considered at levels significantly below the ICH identification thresholds. The acceptable limit for a GTI or PGI is based on several factors, including dose, shelf-life and duration of exposure (i.e., acute vs. chronic). For example, a product with a 1000 mg/day daily dose intended for chronic use may require (based on a TTC of 1.5 µg/day), a maximum acceptable limit for the PGI degradant of 1.5 ppm at the end

of the shelf-life (Table 2).¹ If the degradant is confirmed to be genotoxic, this could then require a lower limit (i.e., <TTC) at product release and a thorough understanding of degradation kinetics to ensure the quality of the API and the drug product (DP) to the end of the shelf-life.

While analytical methodology is clearly an important tool in identifying degradants with genotoxic potential, practical limitations restrict the identification of all low-level degradants in the absence of any other information. It is therefore important that practical approaches be developed to predict potential genotoxic degradants to allow focused analytical efforts on actual levels of genotoxic or potentially genotoxic degradants. In developing a predictive approach, it is useful to divide degradants into three categories.

- *Theoretical degradants*: Hypothetical degradants that are postulated, in-silico and in-cerebro, in the absence of any stability/degradation data.
- *Potential degradants*: Degradants observed during stress studies but not necessarily relevant to the intended storage conditions. Stress studies are designed to produce all potential degradants under the conditions used. Stress studies typically produce a range of degradants under a variety of conditions, but many will not be formed during long-term storage of the drug substance or the drug product. Nevertheless, stress studies can contribute to the overall understanding of molecular degradation processes [i.e., the “intrinsic stability” characteristics of the molecule as described in ICH Q1A(R2)].
- *Actual degradants*: Degradants observed in the product under normal storage conditions. Actual degradants are a subset of potential and theoretical degradants, typically seen under the proposed storage and distribution conditions (e.g., 25°C/60% RH) over the proposed shelf-life. Actual degradants are usually observable during stress and accelerated studies, and often determine the shelf-life under the proposed storage conditions.

According to regulatory guidance on impurities [ICH Q3A (R2) and Q3B (R2)], further consideration over and above standard impurities is required for degradants with genotoxic potential. As stated in the impurities, guidelines [ICH Q3A (R2) and Q3B (R2)] “impurities known to be unusually potent or to produce toxic or unexpected pharmacological effects” should be evaluated for analysis and control at potentially lower thresholds on a case-by-case basis. This aspect of the ICH guidance is expanded and discussed more thoroughly in the EMEA GTIs guidance (1,2), which states:

As stated in the Q3A guideline, actual and potential impurities most likely to arise during synthesis, purification and storage of the new drug substance should be identified, based on a sound scientific appraisal of the chemical reactions involved in the synthesis, impurities associated with raw materials that could contribute to the impurity profile of the new drug substance, and possible degradation products. This discussion can be limited to those impurities that might reasonably be expected based on knowledge of the chemical reactions and conditions involved. Guided by existing genotoxicity data or the presence of structural alerts, PGIs should be identified.

From these guidances, it may be inferred that an understanding of which degradants might be “reasonably expected” is important and expected. There are many elements that could assist in identifying an approach that reliably and succinctly predicts significant actual degradants at the long-term storage conditions over the shelf-life of the product. For the

¹It should be noted that this is an extremely conservative assumption, because it is very unlikely that a given patient would consistently ingest samples of the product that were always close to expiration.

purposes of this discussion, the use of theoretical approaches (i.e., in-cerebro and in-silico) and the application of experimental data from stress studies and elevated temperature/humidity studies (i.e., the use of conditions that represent exaggerated storage conditions) will be explored further.

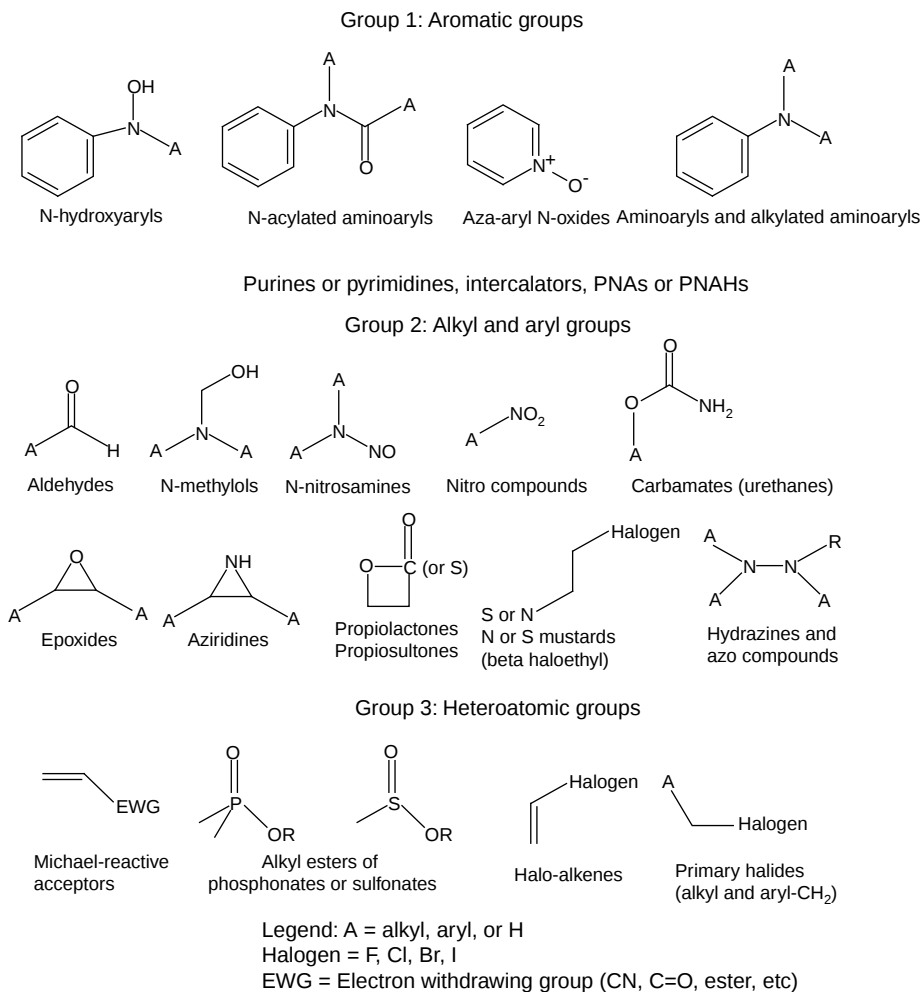


Figure 1 Structurally alerting functional groups that are known to be involved in reactions with DNA.

Using “In Cerebro” and “In Silico” Data for Prediction of Potentially Genotoxic Degradants

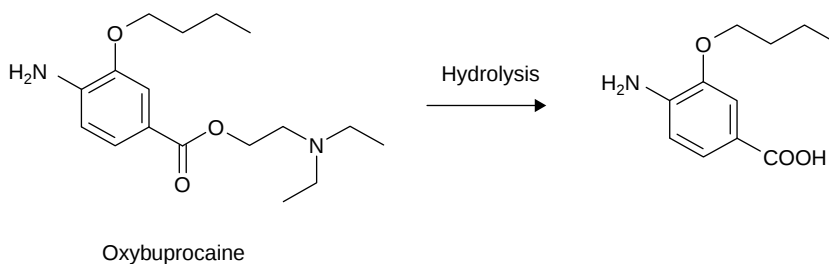
Assessing drug degradants for their genotoxic potential is of growing importance for pharmaceutical development. This is due to the fact that if degradation does lead to the presence of a genotoxin, it takes just a very small degree of degradation to produce levels above the acceptable thresholds (see section “Introduction”) (1,2). Therefore, an understanding of the potential for genotoxins to be formed via drug degradation is important to the development of adequate control strategies. To be most useful, such strategies need to be based on an understanding of the mechanism of degradation, reaction kinetics and the structures of the degradants. Therefore, a systematic approach to the prediction of potential genotoxic degradants should

be expected to become a valuable tool in support of effective and efficient drug development. This section will focus on elucidating the typical mechanisms of formation found for alerting structures in degradants of drug molecules. The two main approaches to the practical evaluation of drug stability and the generation of associated degradant profiles are stress studies and formal (ICH) stability studies (i.e., accelerated stability, long-term stability, and confirmatory photostability studies). Stress studies are of significant value early in development, allowing initial, rapid assessment of the sensitivity of a drug to degradation and the possible range of degradants. An in-depth review of the use of stress studies to evaluate and predict drug stability and degradation profiles can be found throughout the chapters in this book and in the previous edition (8).

Origins for Structural Alerts in Potential Genotoxic Degradants

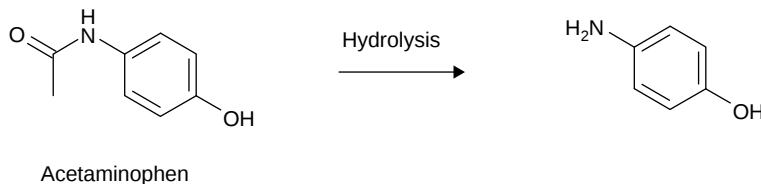
Conceptually, there are two main ways in which an alerting genotoxic drug degradant structure can form.

1. The parent drug that already contains genotoxic alert forms a degradant containing an alerting structure (Figure 1) that is either (i) the same alerting structure or (ii) a different alerting structure. Two examples follow to demonstrate this concept.
 - (i) Degradant with same alerting structure as the parent drug:



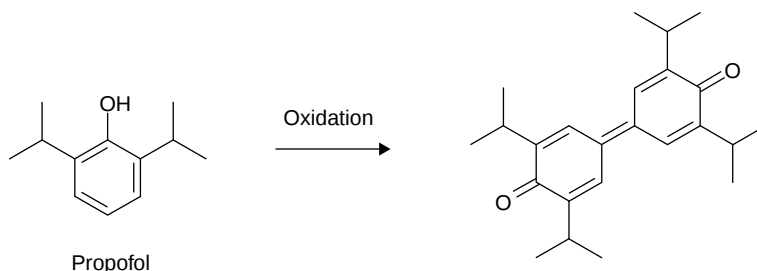
In this case, oxybuprocaine (benoxinate) with a structural alert for aromatic amines forms the corresponding acid via hydrolysis and the structural alert for aromatic amines is retained in the degradant (9). According to EMEA guidance and further described by Dobo et al. (10), impurities (or in this case degradants) with structural alerts that are shared by the parent molecule can be qualified by standardized mutagenicity data obtained with the parent molecule. In addition, however, the chemical constraints for the alerting structure should be similar for both the parent molecule and degradant such that reactivity of the alerting species should not be significantly different. In contrast, a degradant that has a unique alerting structure is not considered qualified with the drug substance.

- (ii) A degradant with a different alerting structure than parent drug is formed:



In this case, acetaminophen, which itself contains a structural alert for *N*-acylated aminoaryls, forms *p*-aminophenol, triggers a structural alert different from the one in the parent structure (an aromatic amine) (11).

2. The parent drug with no alerting structure forms a degradant containing an alerting structure.



In this example, propofol, which lacks a structural alert, degrades via oxidation to a dimeric molecule, containing several conjugated unsaturated carbonyl systems, which are structural alerts for mutagenicity (12).

Evaluation of the CambridgeSoft 3D Drug Degradant Database for the Presence of Genotoxic Structural Alerts

Pharmaceutical drug degradation database (Pharma D3) is web-based, searchable database of drug substances and their respective degradation products,² populated with information various sources in the scientific literature. The database can be searched by substructure, compound number, commercial name, formula and molecular weight for parent compounds or degradants or experimental conditions.³

As of November 2009, Pharma D3 contained 322 unique parent structures and 1021 degradants (13). Bercu et al. (14) have analyzed the parent compounds and their respective degradant structures using ToxtreeTM (v1.51) for the presence of alerting structures (Fig. 1) that are either unique to the impurity or shared with the active pharmaceutical ingredient. ToxtreeTM is open-source software, which can be downloaded from the European Commission's Joint Research Centre.⁴

The software can be used to predict the toxic hazards of chemicals. Included is a decision tree for the prediction of *Salmonella* mutagenicity and carcinogenicity. The decision tree contains a collection of rules, which is in essence a list of structural alerts for mutagenicity. Whereas structures may be analyzed by visual inspection, the collection of rules in ToxtreeTM can serve as a more efficient knowledge base for the identification of mutagenic substances.⁵

The Pharma D3 was found to contain (Table 3):

- 221 alerting structures among the 322 parent molecules (69%),
- 336 alerting structures among the 1021 degradants (33%), and
- 155 alerting unique structures among the 1021 degradants (15%)⁶

²<http://d3.cambridgesoft.com/>

³It is important to note that Pharma 3D does not distinguish between major and minor degradants.

⁴<http://ecb.jrc.ec.europa.eu/qsar/>

⁵It should be noted that ToxtreeTM searches for the presence of alerting structure without regard to the position of the altering structure in the molecule. Thus, ToxTreeTM is a useful tool in defining the frequency with which structural alerts occur in degradation products. More sophisticated programs, such as MultiCaseTM and DEREKTM, take into account not only the presence of an altering structure but also applies more sophisticated rules and machine learning.

⁶When the degradant structures found in Pharma 3D were analyzed by DEREKTM the frequency of unique alerting structures was approximately 8% compared with the 15% found by ToxtreeTM, (14).

Table 3 Toxtree™ Evaluation of the CambridgeSoft 3D Drug Degradant Database Showing the Number of Hits for Structural Alerts in the Parent Drug and in the Corresponding Degradant (the degradants Shown in Bold Collectively Represent Approximately 70% of the Positive Hits for all Degradants in the Database)

Structural Alert and Corresponding ToxTree™ Alert Number	Number of Hits in Parents	Number of Hits in Degradants	Number of Unique Hits in Degradants ^a
Alkyl or benzyl ester of sulfonic or phosphonic acids (SA 2)	3	2	0
N-Methylol derivatives (SA 3)	0	2	2
Monohaloalkenes (SA 4)	0	3	3
Propiolactones and Propiosultones (SA 6)	4	5	4
Epoxides and aziridines (SA 7)	9	17	12
Aliphatic halogens (SA 8)	12	12	6
α , β -Unsaturated carbonyls (SA 10) ^b	79	126	30
Aldehydes (SA 11)	2	40	34
Quinones (SA 12)	11	23	4
Hydrazines (SA 13)	11	12	5
Aliphatic azos and azoxys (SA 14)	0	1	1
Alkyl carbamates and thiocarbamates (SA 16)	5	18	0
Polycyclic aromatic hydrocarbons (SA 18)	0	6	6
Heterocyclic, polycyclic aromatic hydrocarbons (SA 19)	4	15	13
Azide and triazene groups (SA 22)	7	2	0
α , β unsaturated alkoxy (SA 24)	0	1	1
Aromatic nitroso groups (SA 25)	0	2	2
Aromatic ring N-oxides (SA 26)	0	6	6
Nitro aromatics (SA 27)	26	25	6
Primary aromatic amines, Hydroxyl amines and its derived esters (SA 28)	73	93	23
Aromatic mono- and dialkylamines (SA 28)	3	4	2
Aromatic N-acyl amines (SA 28)	5	8	5
Aromatic diazos (SA 29)	0	2	2
Coumarins and Furocoumarins (SA 30)	0	1	1

^aUnique hit in Degradant means that the alerting structure is not shared with the parent drug.

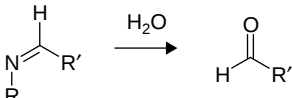
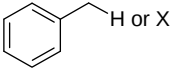
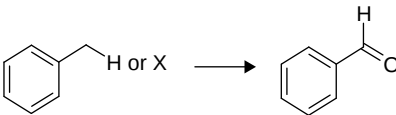
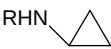
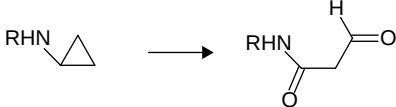
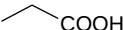
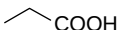
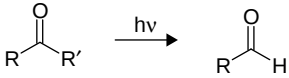
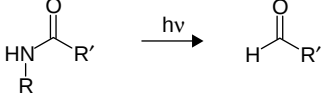
^bThe Toxtree™ analysis of quinones fires a positive hit response for this class, as quinones contain the α , β -unsaturated carbonyl structural element. In this table, the numbers reported for the α , β -unsaturated carbonyl class includes the hits for quinones.

A very informative analysis was obtained by identifying reactions that formed alerting structures not present in the parent molecule (i.e., unique structures). This analysis, (Tables 4–8) showed that five functional groups accounted for almost 70% of the unique alerting structures. Tables 4–8 show the functional groups and chemical reactions responsible for the production of a unique alerting structure and may be used as a predictive tool for the identification of functional groups in that may lead to potentially genotoxic degradants.

The following abbreviations for substituents were used, except where noted otherwise in the tables: R, R', R'', R1, R2, R3 = H or CH_x or C-alkyl or C-heteroatom. In the case of aromatic structures, which are drawn without substituents, the same degradation reaction would be expected to occur in the case of substituted versions, unless the type of substituent on the aromatic ring makes the degradation reaction chemically unlikely.

Because the Pharma D3 database contains only a limited number of drugs, it is clear that Tables 4–8 contain only a subset of all the chemical reactions that can be expected to be relevant for the formation of alerting drug degradants. Nevertheless, the fact that the tables are derived from a compilation of drugs (or drug-like compounds) and related degradation products should make them valuable, as many of the known drug molecules to date contain recurring molecular themes.

Table 4 Reactions Leading to the Production of an Alerting Structure: Aldehydes

Functional Group in Parent Leading to Degradation	Mechanism
Imine 	Hydrolysis 
Benzylalcohols, Benzylamines, Benzylic carbons:  X = OH, NR1R2	Oxidation 
Amino-cyclopropyl 	Photo-oxidation 
Aromatic acetic acid Aromatic substituent 	Oxidation + decarboxylation Aromatic substituent  $\longrightarrow$ Aromatic substituent 
Ketone 	Norrish reaction 
Amide 	Photolysis 

It follows from the preceding discussion that the initial evaluation of a new drug substance degrading to produce a potentially genotoxic degradants should involve interrogation of the parent structure both in-cerebro and in-silico for the presence of alerting structures as well for chemically labile structures that can lead to either (i) preservation of an alerting structure or (ii) creation of an alerting structure through one or more of the reactions shown in Tables 4–8. If this initial assessment does not reveal the potential for a structural alert in each of the theoretical degradants, further consideration should be given to the less commonly observed structural alerts shown in Table 3.

Table 5 Reactions Leading to the Production of an Alerting Structure: α,β Unsaturated Carbonyls

Functional Group in Parent Leading to Degradation	Mechanism
Allyl alcohol	Oxidation
Quarternary ammonium group beta to carbonyl	Hofmann elimination a)
Heteroatom β to carbonyl	b) Substitution/elimination reaction induced with water X = NR₁R₂, OR₁, SR₁R₂ c) 1,4-Elimination
β-Hydroxycarbonyl	Elimination of water
Cyclohexene	Oxidation α to double bond
Phenol	Oxidative dimerization to quinone
Catechol	Oxidation to quinone

Table 6 Reactions Leading to the Production of an Alerting Structure: Primary Aromatic Amine, Hydroxyl Amine and its Derived Esters

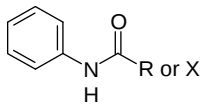
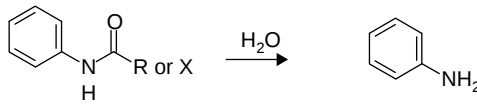
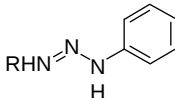
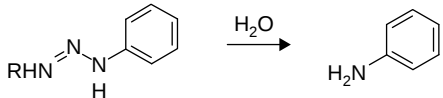
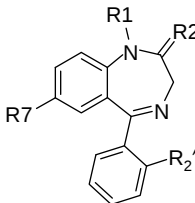
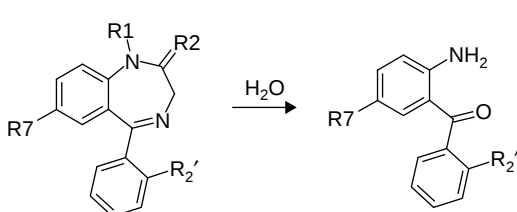
Functional Group in Parent Leading to Degradation	Mechanism
Aromatic <i>N</i>-acyl  R = alkyl X = Heteroatom	Hydrolysis 
Phenyltriazene 	Hydrolysis 
Benzodiazepine  R1, R2, R2', R7 refer to the benzodiazepine substitution nomenclature	Hydrolysis 

Table 7 Reactions Leading to the Production of an Alerting Structure: Epoxides

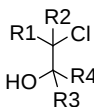
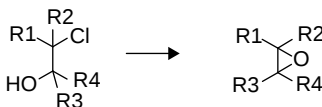
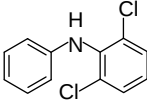
Functional Group in Parent Leading to Degradation	Mechanism
Double bond 	Oxidation 
2-hydroxy-1-chloroalkane 	Cyclization via nucleophilic substitution 

Table 8 Reactions Leading to the Production of an Alerting Structure: Polyaromatic Hydrocarbons

Functional Group in Parent Leading to Degradation	Mechanism
Aromatic chloride in chloro-substituted diphenylamine 	Photodehalogenation reaction, leading to cyclization 

Using Stress Studies for Predicting Rates of Formation of Degradation Products

Stress studies involve the exploration of degradation pathways induced by heat, humidity, a wide pH range in solution, oxidants, and light. Not all of these conditions will be directly relevant or useful for predicting degradants that will actually form during long-term storage. Exposing the API and drug product “as is” to elevated temperatures and humidity conditions could be more appropriate and may provide more relevant and meaningful data (i.e., produce degradants more likely to be seen during long-term storage conditions). The other stress conditions (solution stress conditions of pH, oxidant, and light) are useful in developing stability indicating analytical methods and building the complete picture around the molecule degradation chemistry, and help to build the overall degradation understanding. Recently, evaluation at elevated temperatures and humidity, using modified Arrhenius⁷ equations, has shown to give good prediction of stability at lower temperatures for a range of API and formulated products (15). The activation energy (E_a) is a component of the Arrhenius equation used to quantify the energy requirements for chemical transformations. While E_a may be calculated by experiment, it is not practical to obtain this value for every new drug entity and subsequent formulations. However, the Arrhenius relationship can be used to predict degradation kinetics within the limits of the assumed value of E_a . For example, Davis (16) proposed the approximation that storage for three months at 40°C/75% RH is equivalent to storage for 24 months at room temperature. This approximation assumes an E_a value 25.8 kcal/mol (108.2 kJ/mol) and is the basis for allowing a 24 month shelf-life for generic drugs (submitted under the ANDA regulations) on the basis of acceptable stability data obtained after three months at 40°C/75% RH.

Table 2 shows using an E_a value less than the true value will overestimate the rate of degradation and underestimate the shelf-life when extrapolating degradation data obtained at higher temperatures. Conversely, extrapolation to lower temperature will underestimate the rate of degradation and overestimate the shelf-life if the assumed value of E_a is greater than the true value. Analysis (18) of a database of reactions responsible for drug degradation show that the values of E_a range between 10 and 30 kcal/mol (42–126 kJ/mol) with a mean of approximately 19–20 kcal/mol (80–84 kJ/mol). The current ICH stability guideline (Q1A) recommends 6 months of stability data at accelerated conditions (e.g., 40°C/75% RH for room temperature stored products) and 12 months of long-term testing (e.g., 25°C/60% RH) is sufficient justification to propose a 2-year shelf-life at room temperature. This equates to an E_a value of 17 kcal/mol (71 kJ/mol), which is a more conservative assumption than the value of 25.8 kcal/mol (108.2 kJ/mol) discussed earlier for ANDA applications⁸. While this approach has been demonstrated to be applicable for

⁷Arrhenius equation: $\ln k = \ln A - (E_a/RT)$ where, k , A , E_a , and T are the rate constant, the frequency factor, the activation energy, and the absolute temperature, respectively. The value of the gas constant, R is 1.983 cal/mol/°K (or 8.314 J/mol/°K).

⁸Value of 19 kcal/mol (83 kJ/mol) is also recommended by the USP Pharmaceutical Stability for the Mean Kinetic Temperature in General chapter (1150) and the extrapolation of kinetic data to predict degradation at lower temperatures.

most drug substances, it may not be suitable for some formulations such as topical creams and emulsions due to low melting points and solid form considerations. In addition, an important part of the study design when applying increased temperatures for predicting degradation is that the relative humidity should be kept constant in order to maintain Arrhenius kinetics. Alternatively, a modified Arrhenius equation has been proposed that takes into account the sensitivity of the rate of degradation to the relative humidity (15).

Calculations based on the Arrhenius kinetics [with a conservative estimate for E_a of 17 kcal/mol (71 kJ/mol)] indicate that storage at 60°C/75% RH for one month would be equivalent to approximately 19 months at room temperature and six weeks storage would approximate 28.5 months at room temperature. The relative increases in degradation rates that justify stability studies of shorter duration conducted at elevated temperature are shown in Table 9. The process for assessing whether or not a potential genotoxic degradant is “reasonably expected” or “likely” can include analyzing the elevated temperature/humidity sample(s) using a stability-indicating analytical method (developed from the stress studies). This work would begin the process of flagging those degradants directly relevant to the storage conditions of the API and the drug product.

Combining Information from Stress Studies with in-Cerebro and in-Silico Techniques to Understand the Risk of Producing Potentially Genotoxic Degradants

It is important that predictive or theoretical approaches to understanding of potential degradation pathways be combined with results from stressed studies and accelerated stability studies to enable a more accurate prediction of the potential for production of low levels of potential genotoxic degradation products. Awareness of theoretical degradants that contain structural alerts prior to conducting stressed degradation and/or real time stability studies can be an important factor in the design of these studies as it alerts the analytical chemist to look for these theoretical degradants among the major degradants during stressed studies. Leveraging multiple tools (predictions, analytical, chemistry, stressed studies) will increase the overall understanding of the degradation knowledge space and focus analytical attention on the low-level detection of degradants on a case-by-case basis. This science-based approach is preferred to the development of a battery of highly sensitive, specialized methods applied to all samples subjected to stress conditions because it focuses attention on the high-risk cases where the presence of potentially genotoxic degradants is likely. Thus, combining accelerated stability and stress testing with in-cerebro and in-silico approaches allows for the construction of a rational, risk management argument to understand and assess the probability of producing potentially genotoxic degradants.

Table 9 Rates of Degradation (Relative to 25°C; Assuming Arrhenius Kinetics and Constant Humidity) and Energies of Activation (E_a) of 12, 15, and 20 kcal/mol

Temperature (°C)	Relative Rate Assume $E_a = 12$ kcal/mol (50 kJ/mol)	Relative Rate Assume $E_a = 15$ kcal/mol (63 kJ/mol)	Relative Rate Assume $E_a = 17$ kcal/mol (71 kJ/mol)	Relative Rate Assume $E_a = 20$ kcal/mol (84 kJ/mol)
25	1.00	1.00	1.00	1.00
30	1.39	1.52	1.61	1.75
40	2.62	3.37	3.96	5.05
50	4.78	7.10	9.23	13.65
60	8.36	14.33	20.44	34.79
70	14.20	27.74	43.22	83.98

ANALYTICAL CONSIDERATIONS

Table 2 highlights a number of important considerations that set the determination of genotoxic degradants apart from the determination of “ordinary” (nongenotoxic) degradants. The allowable daily intake (and thus the concentration) of a GTI is generally less than that encountered for ordinary degradants (Table 1). For example, ICH Q3A (R2) defines the lowest reportable concentration for an ordinary impurity as 0.03% (300 ppm) in the drug substance. In sharp contrast, the necessary detection limits for genotoxic degradants may be one to two orders of magnitude lower, requiring analytical methodology with greater sensitivity (Table 2). The need for greater sensitivity arises from the fact that method precision decreases with decreasing analyte concentration (17). Thus a chromatographic peak at the quantitation limit ($S:N = 10:1$) will be indistinguishable from the noise at concentrations ten times less. Approaches to increase the sensitivity, without changing the technique (e.g., HPLC-UV), may involve changes in the sample preparation procedure to increase the concentration of the degradants in the sample or derivatization to increase the sensitivity. Both these procedures come at the potential expense of speed and selectivity; and alternative techniques (e.g., LC-MS/MS) may be necessary to achieve the necessary sensitivity and selectivity.

A second important difference in the analysis of genotoxic degradants compared with ordinary (nongenotoxic) degradants is the allowable limits (s-TTC or TTC, Table 1) for a genotoxic degradant in both the drug substance and the DP are the same. In contrast, the ICH qualification limits [ICH Q3A (R2) and Q3B (R2)] for nongenotoxic degradants in the DP are higher than those in the drug substance. The original rationale behind the ICH impurities guidelines to allow higher limits for degradants in the DP compared with drug substance is that degradants were considered less reactive and potentially less toxic.⁹ However, this chapter has demonstrated that genotoxic or potentially genotoxic compounds can be produced as a result of degradation of the drug substance in the API or in the drug product.

Frequently, analysis of degradants in the drug product can be more challenging than in the drug substance, due to the presence of excipients as well as additives such as preservatives and antioxidants and their corresponding degradants, all of which may interfere with the analysis. A result of the differences in the ICH limits for degradants in the drug substance compared with the drug product is that less sensitive methods are generally required for the drug product. In addition to the technical considerations, having lower limits for degradants in the drug substance provides some leeway for additional degradation to occur without the degradant exceeding its limit in the drug product. No such leeway exists for genotoxic degradants (Table 2) and, as discussed previously in section “Analytical Considerations,” more sensitive methods and potentially lower limits may be necessary for genotoxic degradants in the drug substance to avoid the possibility of drug-product failure during manufacture and storage.

Thus, analytical methodology with greater sensitivity, better precision at lower analyte concentrations and higher specificity may be needed for the quantitative determination of genotoxic degradants in the drug substance and DP. Consequently, the analytical chemist charged with the determination of GTI may be faced with the need to use techniques more commonly used in trace analysis of drugs in biological fluids, such as LC-MS/MS. Whereas it is not recommended here that such techniques be used routinely in stress testing, they may be necessary if a presence of a potentially genotoxic degradant is suspected.

CONCLUSIONS

At present there is no consistency or agreement within the pharmaceutical community regarding the factors to include when trying to understand the risk that potential genotoxic degradants may be produced in the drug substance or in the DP during routine storage. This chapter has

⁹Unpublished discussions of the ICH expert working group on impurities in the drug substance and drug product (Q3A and Q3B).

attempted to provide potential approaches and factors to consider when predicting and detecting the presence of low-level genotoxic degradants. These approaches combine theoretical knowledge obtained in-cerebro and in-silico with stress studies to assess the risk of a genotoxic or potentially genotoxic degradant being present above the allowable daily limit at the end of the shelf-life and avoids the routine use of highly sensitive, specialized analytical method for all stress studies. Such methods should be reserved for those cases where it is known or highly likely that a potentially genotoxic or known genotoxic degradant will be produced.

Software programs such as ToxTree™, MultiCase™, and DEREK™ have become important tools in the evaluation of chemical structures for potential genotoxicity. This chapter has highlighted how some of the currently available information for evaluating alerting structures found in drug degradants can be used towards classifying structural elements in parent molecules that have the potential to lead to alerting structures during degradation.

Continued interdisciplinary collaboration between toxicologists, analytical chemists, process chemists, pharmaceutical scientists, and software experts is essential for the expansion of knowledge about details of drug degradation and its implication for toxicity.

REFERENCES

1. Guideline on the limits of genotoxic impurities. European Medicines Agency, Committee for Medicinal Products for Human Use, 2006.
2. Questions and answers on the CHMP guideline on the limits of genotoxic impurities. European Medicines Agency, Committee for Medicinal Products for Human Use Safety Working Party, 2008.
3. Guidance for Industry (draft), Genotoxic and carcinogenic impurities in drug substances and drug products: Recommended approaches. Food and Drug Administration, Center for Drug Evaluation and Research 2008 (Dec).
4. Müller L, Mauthe RJ, Riley CM, et al. A rationale for determining, testing, and controlling specific impurities in pharmaceuticals that possess potential for genotoxicity. *Reg Toxicol Pharmacol* 2006; 44: 198–211.
5. Pierson D. Approaches to assessment testing decisions, and analytical determination of genotoxic impurities in drug substances. *Org Proc Res Dev* 2009; 13: 285–91.
6. Snodin D, Vudathala GK. Genotoxic impurities: a case for regulatory rethink. *AAPS News Magazine* Feb. 2009: 20–7.
7. Rulis AM. Establishing a threshold of regulation. In: Bonin JJ and Stevenson DE, Eds. *Risk Assessment In Setting National Priorities*. New York: Plenum, 1989: 271–8.
8. Baertschi SW, ed. *Pharmaceutical Stress Testing: Predicting Drug Degradation*. 1st edn. New York: Informa Healthcare, 2007.
9. El-Grindy A. First derivative spectroscopy and LC determination of benoxinate hydrochloride and its degradation Products. *J Pharm Biomed Anal* 2000; 22: 215–34.
10. Dobo KL, Greene N, Cyr MO, Caron S, Ku WW. The application of structure-based assessment to support safety and chemistry diligence to manage genotoxic impurities in active pharmaceutical ingredients during drug development. *Regul Toxicol Pharmacol* 2006; 44: 282–93.
11. Florey K, ed. *Analytical Profiles of Drug Substances*. New York: Academic, 1974: 39.
12. Baker MT, Gregroson MS, Martin SM, Buettner GR. *Crit Car Med* 2003; 31: 787–92.
13. Benigni R, Bossa C. Structure alerts for carcinogenicity, and the Salmonella assay system: a novel insight through the chemical relational databases technology. *Mutat Res* 2008; 659: 248–61.
14. Raillard SP, Bercu J, Baertschi SW, Riley CM. Prediction of drug degradation pathways leading to structural alerts for potential genotoxic impurities. *Org Proc Res Dev* 2010; 14: 1015–20.
15. Waterman KC, Adami RC. Accelerated aging: Prediction of chemical stability of pharmaceuticals. *Int J Pharm* 2005; 293: 101–25.
16. Davis, JS, FDA/ASQC Seminar, Chicago, IL, March 11, 1991.
17. Riley CM. Statistical parameters and analytical figures of merit, in development and validation of analytical methods, progress. In: Riley CM and Rosanske TW, eds. *Pharmaceutical and Biomedical Analysis*. Vol. 3. Oxford: Elsevier, 1996: 15–71.
18. Kennon L. Use of models in determining chemical pharmaceutical stability. *J Pharm Sci* 1964; 53: 815–18.

20 | The power of computational chemistry to leverage stress testing of pharmaceuticals

Donald B. Boyd and Thomas R. Sharp

INTRODUCTION

It is a fundamental tenet of chemistry that the structural formula of any compound contains coded within it all that compound's chemical, physical and biological properties. (From Ref. 1)

The above quotation states an article of faith that most chemists will generally accept as true. But do we yet understand "structure" sufficiently that we no longer need to perform experimentation? Most would likely agree "not yet."

This chapter describes an area of research, finding new uses every day. The tools of computational chemistry—computers and software—have become so ubiquitous that there are few chemists left today who have not heard of them or used them. However, the reader may reasonably ask "Why is there a chapter on computational chemistry in a book on stress testing of pharmaceutically interesting compounds?" The reason does not lie in a vast number of papers published on the subject. Indeed, relatively little work has been published in this area thus far. So what is the reason? The objective of this chapter is to increase awareness on how computational chemistry can be used by pharmaceutical chemists to help confront some of the research problems they face in stress-testing research. Hence, the purpose of this chapter [and the chapter by one of us appearing in the first edition of this book (2)] is to proselytize for increased use of the techniques of computational chemistry now available. The first edition chapter (2) introduced concepts and tools, making sense of the specialized language common to the cognoscenti. The chapter in this edition seeks to reinforce and expand upon some of the computational approaches that are available to the laboratory scientist today. Applications of the approaches are reported to illustrate the widening scope of the technology.

Computational chemistry can be a help to the experimentalist in a number of ways. These include (i) visualizing and quantifying information about molecular structure, (ii) answering questions pertaining to electronic structure and hence reaction mechanisms, and (iii) honing questions that can be investigated experimentally. In effect, the computer is used for learning about atomic and molecular phenomena, just like with other laboratory instruments. A key point that pharmaceutical chemists should bear in mind is that computational chemistry's goal is not to replace experiment but to supplement what can be learned experimentally. Any theoretician who asserts otherwise is being naïve, or worse.

Computational chemists have made their software tools increasingly easier to use so that an enlarging population of scientists may use them conveniently. Generally, these software advances entail graphical user interfaces (GUIs), which enable individuals to use a simple paradigm of point-and-click, pull-down menus, and pop-up dialog boxes to build molecular models, set up calculations, and display the results on a computer screen. At the same time, because the underlying theoretical models can be quite sophisticated and complex, opportunities for inappropriate use or misunderstandings or misinterpretation abound. In his book, Young (3) quotes Karl Irikura, a scientist at the National Institute of Standards and Technology, reminding us that "anyone can do calculations nowadays. Anyone can also operate a scalpel. That does not mean all our medical problems are solved."

In this chapter, we will suggest some ways to avoid common difficulties. We also try to present an intellectually honest and practical guide. We want to show not only where the power of the technology can be applied, but also the way around potential traps that could ensnare new users. By the end of this chapter, the readers will hopefully feel empowered to investigate the methodologies in applications to some of their immediate research problems. The tools to be described can give the chemist a competitive advantage in solving problems and enhancing publishable work.

WHAT IS COMPUTATIONAL CHEMISTRY?

Computational chemistry emerged as a distinct discipline about 30 years ago. The terms “computational chemistry” or “calculational chemistry” started being used in the literature in the 1970s, but a terminology did not become established until 1980 when the *Journal of Computational Chemistry* was founded. Essentially all research-based pharmaceutical and chemical companies have come to recognize its value and have established groups of computational chemists (also called molecular modelers or cheminformatics experts). There are many ways to illustrate the growth of the field. Approximately 15 years ago, 13% of papers in the chemical literature contained some computational chemistry work (4). The percentage was steadily increasing. Another analysis (5) showed that in certain journals, such as *The Journal of the American Chemical Society* and *Journal of Medicinal Chemistry*, about 25% of their papers contained computational chemistry results. Citation analysis of computational chemistry publications catalogued by the Chemical Abstracts Service (6) shows a similar trend, as well as an examination of this topic in the preface of Bachrach’s book (7).

Most published definitions embrace an overlapping set of ideas. Computational chemistry has been described as consisting of those aspects of chemical research that are expedited or rendered practical by computers (8). Such a scope is so broad that it includes most molecular science laboratories where modern instruments are built with programmed or programmable computers to collect and mathematically manipulate data. A narrower definition of computational chemistry was published in 1985 (9): “quantitative modeling of chemical behavior on a computer by the formalisms of theoretical chemistry.” Based on his popular textbook, Andrew Leach (10) views computational chemistry as encompassing “not only quantum mechanics, but also molecular mechanics, [energy] minimization, [molecular] simulations, conformational analysis, and other computer-based methods for understanding and predicting the behavior of molecular systems.” The International Union of Pure and Applied Chemistry (IUPAC) (11) agreed on a definition of computational chemistry about 15 years after the term was already well established in the chemical lexicon: “A discipline using mathematical methods for the calculation of molecular properties or for the simulation of molecular behavior. It also includes, for example, synthesis planning, database searching, combinatorial library manipulation.” The open-ended second part of this statement reflects the disparate opinions of the IUPAC committee and others who contributed comments.

Some quantum theoreticians naturally would like to see computational chemistry as a subset of their field (12). However, today the number of scientists employed as computational chemists well exceeds the number employed as theoreticians (13). Computational chemistry includes applications of theoretical chemistry, but theoretical chemistry and computational chemistry are not synonymous. Theoretical chemistry involves development of mathematical expressions that model physical reality; as such, some of theoretical chemistry entails quantum mechanics. Computational chemistry, on the other hand, involves use of computers on which theoretical and many other algorithms have been programmed.

The scope of computational chemistry can be inferred from the methodologies it encompasses. Some of the more common tools include computer graphics, molecular modeling, quantum chemistry, molecular mechanics, statistical analysis of structure–property relationships, and data management (informatics). As with any dynamic field of research, computational chemistry is growing, new methodologies are being developed, and the scope is evolving. For instance, 30 years ago, quantum chemistry was by far the most used approach in computational chemistry, but 10 years later force field calculations (molecular mechanics and molecular simulations) had grown to be the most used approach (14).

INFORMATION RESOURCES

We will not attempt any degree of mathematical rigor here. We believe there are two styles of thinking—the one in which one conceptualizes a phenomenon visually, and the one in which the mere examination of a mathematical equation tells all. Einstein, purportedly, was actually of

the former visual way of thinking (15) and subsequently sought mathematical ways to describe his visualizations. Individuals who can visualize directly from the equations, we believe, are rather rare. Quantum mechanics applied to chemistry necessarily relies on a detailed understanding and implementation of those equations. On such implementations, most of us must rely upon others to have done the implementation correctly as a matter of faith. Our approach here will be descriptive and phenomenological, will rely on that article of faith and will defer anyone interested in the hard-core mathematics to other resources such as Szabo and Ostland (16), Cook (17), and McMahon (18). Hundreds of books in the field published up to 2000 have been compiled (19). Older textbooks include those of Hehre et al. (20) and Pople and Beveridge (21). Current textbooks include those by Cramer (22) and Lewars (23). In particular, an excellent background primer on essential mathematical concepts is available in the appendix of Hinchliffe's book (24). The first chapter of Bachrach's book (7) describes the signatory steps without overly belaboring the mathematics, and explains the origins of much of the common computational chemistry jargon.

HISTORICAL CONTEXT OF PEOPLE AND EVENTS

"... If there is a mortal sin ..., it is the teaching of science without connecting to the history of science.... The lack of historical perspective was dramatically demonstrated... by the student who asked if Galileo and Einstein ever met." (From Ref. 25)

Computational chemistry, as any other human endeavor, did not happen in the absence of people. And people and personalities have always been an interesting part of study in and of itself. A number of books have appeared commenting on the people, places, and interactions of the seminal figures of this field, such as the recent popular Einstein biography (26), and the Lindley (18) and Jones (27) treatments. A listing of names important to the field has been published (6). Bachrach (7) includes interviews of living "historical" figures in the field in order to capture some of the personal essence of the field. A history of the Gordon Research Conference on Computational Chemistry is a record of who have been key figures in the growth of the field (28). These references should provide an entry into the subject.

The following list of some seminal events in the development of quantum mechanics attempts to capture a sense of the timeline and people involved, not necessarily in strict chronological order or order of importance. It is not a complete list and neglects molecular mechanics entirely. Discussing and defining the entries in the list could serve as the better part of a final examination in a computational chemistry course.

- Photoelectron effect (1905)
- Bohr model of the hydrogen atom (1913)
- de Broglie wave-particle duality of subatomic particles (1923)
- Pauli exclusion principle (1925)
- Schrödinger equation (1926)
- Heisenberg uncertainty principle (1927)
- Born–Oppenheimer approximation (1927)
- Heitler–London linear combination of atomic–molecular orbital (MO) theory (1927)
- Hartree one-electron treatment (1928)
- Variational principle (1930)
- Hückel pi-electron theory (1931)
- Hartree–Fock–Roothaan–Hall method (1951)
- Pariser–Parr–Pople self-consistent-field pi-electron calculations (1953)
- Basis set definition and improvement (1960s to 1990s)
- Semiempirical MO methods (1962–1965)
- Density functional theory (1965)

- Electron correlation methods such as perturbation theory (1934), coupled clusters (1966), and configuration interaction (1970s)
- Composite energy (also called multilevel) methods such as Pople's Gaussian methods (1993)

The above events pertain to the formulation of the theory of quantum mechanics, which attempts to describe the electronic structure of atoms and molecules. The development of computer programs to implement the mathematics of the theory is also important from a historical perspective. In the interest of brevity, we mention only two representative highlights. One was the writing and distribution of MOPAC (molecular orbital package), which is a program for semiempirical MO calculations. Its significance is that it was the first widely available quantum mechanical program that could predict the three-dimensional structures of organic molecules including pharmaceuticals. Another highlight pertains to Gaussian, a comprehensive quantum mechanical program. The significance is that the program was commercialized in 1987, representative of the fact that academic scientists were becoming aware in the 1980s that there was a market for the software that numerous graduate students and postdoctoral fellows had been writing since the 1970s.

In the realm of molecular mechanics, molecules are treated as if bonded atoms are connected by springs. The springs are represented mathematically. Likewise, bond angles and torsional angles (about rotatable bonds) are represented by potential energy functions. The object of optimizing a molecular "geometry" is to find the three-dimensional structure where all the bond lengths, bond angles, and torsional angles are as close as possible to their minimum energy (most stable) configurations. The idea behind molecular mechanics can be traced back to the 1930s and 1940s when scientists realized that bond lengths and bond angles have certain "natural" values and scientists were tackling problems in physical organic chemistry and vibrational spectroscopy. When computers became available, molecular mechanics calculations became much easier (29). The set of potential energy functions and associated parameters is called a force field. The first force field for proteins was implemented on a computer in 1969 (30). Force fields for organic molecules became more refined over time taking into account subtle connections between the bends and stretches in a molecule. A landmark was the development of the MM2 program in the 1970s (31).

Places are important in the history of a discipline. Compilations have also been published on places important to the development of computational chemistry (32,33,34,35,36).

TOOLS OF THE TRADE

Computer Graphics

An important tool of computational chemistry is computer graphics. It provides the interface between the user and the computer. Molecular structure is the universal language of chemists. Most humans interact visually with their surroundings. Therefore, an important advancement is when a user enters a molecular structure into the computer, and visualizes both the input structure to a computation and the results of that computation.

The simplest graphical depiction of the structure of a molecule has lines, representing bonds, and connecting points representing atoms. Molecular models can also be shown as balls (atoms) and sticks (covalent bonds) or simply as tubes (covalent bonds). Space-filling models can be produced, representing the modern equivalent of CPK (Corey–Pauling–Koltun) space-filling hand-held models. More complex graphics representing molecules as solid objects with colored or translucent surfaces convey not only the shape of the molecule but also electronic properties that pertain to each region of the molecule. Examples are shown in Figure 1. The complexity of three-dimensional molecular models makes high resolution, color, and stereo graphics in a computer visualization highly desirable if not essential.

Program options for a calculation can be set up through a GUI, and after a calculation is complete, the results may be examined visually (37). Quantum chemical and molecular

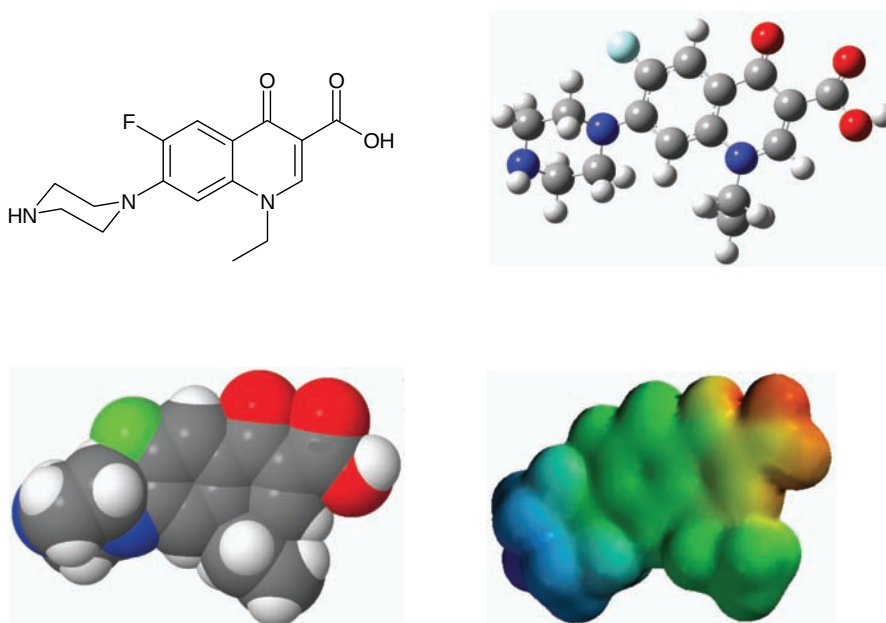


Figure 1 Different graphical representations of a sample molecule: the fluoroquinolone antibacterial agent norfloxacin. *Upper left*, standard 2D chemical diagram, in common use by organic chemists. *Upper right*, ball-and-stick representation with implications of 3D (produced by quantum mechanical geometry optimization), using standard color-coding for identifying the elements. *Lower left*, CPK space-filling representation. *Lower right*, molecular electrostatic potential energy map, projected onto a surface approximating the van der Waals volume of the molecule. Red signifies negative charge, blue positive charge, green slightly positive, and yellow neutral charge. Charge distributions for the neutral and zwitterionic forms of the molecule would be quantitatively different, but qualitatively similar; the carboxylate would be very negative and the amines would be very positive.

simulation methods are computationally intense and generate large quantities of data. Computer visualization of the output helps make the data meaningful. We currently take for granted the facility we presently have available for representing chemical structures in the computer. Both Todd (38) and Johnson (39) emphasize that a significant innovation associated with the early days of developing such systems was the effort required to represent molecules (graphically and otherwise) in the computer. It is interesting to contrast Johnson's thoughts (39), regarding limitations to progress in computational chemistry because of computer power in 1985 to today when modern hardware and software capabilities are commonly available and taken for granted.

Databases

Two-dimensional and three-dimensional types of molecular structure databases are available. The 2D type stores atoms (chemical elements) and connectivity information (i.e., which atoms are bonded to which one in a molecule). The 3D type stores, in addition, the x , y , z Cartesian coordinates of each atom in a molecule. The databases are managed using software that allows fast registration of new structures, fast retrieval of previously stored compounds, and fast substructure searching (40,41). Whereas 2D databases of compounds play an invaluable role in pharmaceutical discovery research, a computational chemistry calculation on a molecule often requires its 3D structure. (Indeed, the 3D structure of a molecule is of paramount importance in defining its pharmaceutical relevance.) A variety of different structural representation formats are available. Many computational chemistry software packages have their own (sometimes

proprietary) internal storage formats, but can read most of the standard ones. The various graphic structure-drawing packages can generate these generic formats in addition to their own proprietary formats. The MDL (now Accelrys) molecular file (molfile) and structure-data file (SDF) are examples of file formats.

An excellent starting point for such a calculation is sometimes one of the molecules in the Cambridge Structural Database (CSD), a 3D database. The CSD (42,43,44) presently has atomic coordinates and other information for over 500,000 small organic and organometallic compounds, most of which have been solved by x-ray crystallography. The number of structures in the database is growing by about 10% per year. Access to the database requires a subscription (45). Besides chemical structure databases, both proprietary and public databases contain a wealth of numerical and textual information pertinent to chemistry. The National Library of Medicine/National Institutes of Health's PubChem on-line database has 2D and 3D structures of a large number of compounds available in SDF format. Viewer software is also available on the PubChem website (46).

Molecular Mechanics (Force Field Modeling)

In molecular mechanics, molecular dynamics, and Monte Carlo simulations, an empirical force field describes how the energy of a molecule changes as a function of bond length l , bond angle θ , and torsional angle ϕ . The theory is chemically intuitive, and the energy functions and parameters are set empirically to reproduce experimental molecular geometries and relative energies as closely as possible.

A set of classical energy terms and associated parameters is called a force field (47). The energy is a combination of bond stretching/compression, bond angle bending, torsional twisting, van der Waals interactions, and electrostatic forces that tend to hold the atoms at their equilibrium positions (48,49,50). Simpler force fields assume harmonic force constants for stretching and bending movements. In more sophisticated force fields, additional terms take into account anharmonicity of bond stretching and angle bending and special electronic effects. A van der Waals energy term, which represents atoms as squishy spheres occupying volumes, is attractive at long range and repulsive at distances shorter than the sum of the van der Waals radii. The force field parameters are calibrated to reproduce experimental geometries, relative energies, and sometimes other molecular properties.

In molecular mechanics, the objective is to compute the energy of a molecular structure and/or to minimize the energy as a function of geometrical degrees of freedom, that is, the potential energy surface. The computed energy reflects how far the geometrical arrangement of atoms deviates from an idealized bonding situation (such as would be the case when there is no strain in any bond). A better geometry has a lower energy. Well-established optimization methods are used for energy minimization.

The molecular mechanics model is conceptually simple, gives very accurate three-dimensional structures for common organic compounds, and provides information at least an order of magnitude faster than do quantum mechanics computational methods. These strengths make molecular mechanics well suited for treating both small and large molecules. Limitations are that it requires empirical parameters to describe the strength of each "spring," and predictions depend on the care with which parameterization has been done. The analogy in quantum chemistry is that predictions depend on the adequacy of the basis set and level of theory. (More on these topics later.)

Ten to twenty force fields are in wide use. Some have simpler and/or fewer potential energy terms such that they can be used to model large biomolecules. Other force fields are carefully parameterized and have extra terms to reproduce more detailed molecular effects that have been observed experimentally. Examples of the latter are the MM2 (51,52) MM3 (53,54,55) and MM4 (56,57,58,59,60) methods of Allinger. The Allinger force fields work very well on common kinds of organic molecules. A force field that was designed specifically for pharmaceutically relevant molecules is the Merck molecular force field (MMFF) (61,62,63,64,65,66,67,68).

Compared to other force fields, the Allinger and Merck force fields give the best overall results for molecular geometries and conformational energies (69). MM2* and MM3* are close variants of MM2 and MM3 as used in the MacroModel program and MOE (see below). A nice stepwise hands-on progression to developing an understanding of molecular mechanics principles is given in Donald Rogers' book (70). (He also develops quantum mechanical principles similarly.)

Some of the more approximate or specialized force fields encountered in the literature include CFF, CHEM-X, COSMIC, CVFF, DREIDING, MMX, SHAPES, TRIPOS, VALBOND, and UFF. These force fields generally are designed to work on isolated (unsolvated) molecules. Well-known force fields for modeling proteins and nucleic acids include AMBER, CHARMM, ECEPP, GROMOS, and OPLS. Some of these latter force fields use a minimal number of potential energy functions so as to be fast enough to treat biomacromolecules in long molecular simulations. The force fields for biomolecules are typically parameterized to handle molecules in a solvent (usually water) bath so as to model a more realistic situation.

Molecular dynamics simulations give information about the variation in structure and energy of a molecule over an interval of time (71,72). In molecular dynamics, each atom moves according to Newton's equations of motion for classical particles. Thus, at each time step, atoms under the most strain move fastest and farthest. To solve Newton's equations satisfactorily requires extremely short time steps, typically only 1 fs (10^{-15} s). If the time increment is too long, atoms can come too close in each step, and the energy of interaction from the force field gets so high that the system becomes unstable and erratic in subsequent steps (73).

A concept useful for obtaining free energies is the thermodynamic cycle. The first law of thermodynamics states that the work done by a system changing between two states is the same for every adiabatic path between the states. Free energy is a state function, so the change in free energy must be the same regardless of how you go around the cycle. There may be situations where it is too difficult to compute the free energy for a reaction or transformation along one route, whereas it may be relatively easy to compute the free energies for a multistep alternate route. Calculations can thus be set up to run the alternate steps. Then the results can be combined to yield the free energy change for the originally desired step.

Thermodynamic cycles are used in molecular dynamics simulations for computing accurate relative free energies. Free energy perturbation calculations simulate the conversion of one molecule into a similar one (74,75). By changing the force field parameters in tiny increments from those of one chemical structure to those for another similar structure (e.g., replacing a methyl substituent with a chloro), the change in free energy can be accurately estimated. The incremental changes must be small so that the molecular system stays in equilibrium. In practice, this means the calculations are demanding of computer time and the alchemical transformations must be rather modest (76). Hence, for many industrial applications, free energy perturbation calculations have limited applicability [but there are notable exceptions (77)].

In molecular dynamics calculations, the molecular system is moving along a time course (trajectory). In Monte Carlo type molecular simulations, the system takes random jumps throughout configuration space (78). A large number of more or less randomly chosen configurations are considered. The energy of each is computed. An ensemble average of the computed energies is used to calculate thermodynamic properties via statistical mechanics.

Quantum Mechanical Modeling

Stress testing involves speeding up the rate at which molecules degrade so the reactions can be conveniently studied in the time frame of pharmaceutical development. Computational chemistry allows us to model reactions at the speed of computers. Since bonds are forming, breaking, or rearranging during reactions (79), electronic structure calculations are usually better at modeling a system than force field methods because chemical bonding is all about the electronic structure of the molecule. [See, however, an exception (80).]

As noted earlier, theoretical chemistry had its origins in the quantum mechanics developed in the 1920s. The early calculations were done on mechanical adding machines. It was not

until the 1950s that the early (slow) computers became available to research chemists. Quantum mechanics is embodied in the famous Schrödinger equation, $\mathbf{H}\Psi = E\Psi$, from physics, where $\mathbf{H}$ is the Hamiltonian mathematical “operator” having kinetic and potential energy terms, Ψ is the wave function describing the positions of particles (electrons and nuclei) in a system, and E is the energy of the system (44). This equation is not a conventional algebraic equation—dividing both sides by Ψ does not solve the problem—but rather, it is an Eigen equation. In effect, the Hamiltonian operator is a mathematical expression of partial derivatives and distance-dependent terms that when applied to a wave function extracts a property (observable) from the wave function and gives back the wave function unchanged. The Hamiltonian operator, and therefore the Schrödinger equation, can be written explicitly and solved analytically only for systems that contain a single electron, such as the hydrogen atom and H_2^+ .

In MO theory, which is the most common chemical implementation of quantum mechanics, electrons are distributed around the atomic nuclei until they reach a so-called self-consistent field (SCF)—that is, until the attractive and repulsive forces between all the particles (electrons and nuclei) are in a steady state, and the energy is at a minimum. An SCF calculation yields the electronic wave function Ψ_e , the electronic motion being separable from nuclear motion, according to the Born–Oppenheimer approximation. The latter assumes that the nuclei of atoms are so much more massive than the electrons and do not move, such that their motions can be treated by separate equations. The electrons zip around a (temporarily fixed) scaffold of nuclei. The electronic wave function describes the distribution of electron density in a molecule and is what is usually referred to in the literature and in the rest of this chapter.

Hartree–Fock Theory

After establishing that the Schrödinger equation is not analytically solvable for any but the simplest systems, the progress and evolution of quantum chemistry can be tracked by a series of successively less crude approximations, aimed at improving the understanding of the subject and made possible by improvements in computing capabilities. The simplest MO approach, and essentially the first, is the Hückel approximation. Although not used in the current research literature, it serves as a useful conceptual starting point. The primary, and limiting, assumption of Hückel’s approach is that the important chemistry involves the π electrons of conjugated double or aromatic bonds. Basic Hückel theory applies specifically to planar conjugated systems. Matrix algebra facilitated the calculations, but the Hückel approximations were made simply for the expedient reason that necessary computer power for anything more than these approximations was not available at the time.

Hartree’s simplification was to treat the wave function as a combination of orbitals each holding a single electron, influenced by a constant composite electrostatic field produced by all the other electrons and nuclear charge of a molecule. This approximation allowed writing a set of equations—one for each electron influenced by the composite field of the other electrons and nuclei. The equations served as a starting approximation to iteratively calculate better and better approximations to the real situation—the SCF approach. The LCAO (linear combination of atomic orbital) approach, resulting in the Hartree–Fock–Roothaan–Hall matrix equations, permitted calculations on substantially larger molecules than the hydrogen atom and H_2^+ . Thus, significant progress in predicting molecular properties was made, in spite of the fact that the theory did not fully account for electron correlation (the ability of other electrons to respond essentially instantaneously to changes in each other’s environment).

The Semiempirical Approach

In semiempirical calculations (81) additional approximations are made, such as treating only the outermost (i.e., valence) electrons while assuming the core (inner) electrons simply neutralize part of the nuclear charge. Of the remaining integrals, the easier ones are computed exactly and the rest are approximated or treated as parameters chosen such that the output of a calculation will be reasonably close to experimental values of target properties of a selected set of

compounds (often small organics). The main advantage of semiempirical calculations is that they are considerably faster (seconds to minutes of compute time on a modern benchtop/laptop computer for small molecules) than *ab initio* (from first principles) calculations (minutes to hours to days). The main disadvantage is that the semiempirical results are generally less accurate than *ab initio* results. If absolute accuracy or precise intermolecular comparisons are required, *ab initio* calculation must be done. However, if relative results, with likely canceling out of systematic errors, are sufficient, semiempirical methods can be more practical and cost effective.

Implementations of semiempirical methods include MINDO/3 (modified intermediate neglect of differential overlap, third parameterization), MNDO [modified neglect of diatomic differential overlap (82,83,84)] AM1 [Austin model 1 (85,86,87,88)] and PM3 [parametric method 3 (89)]. The last major effort from the Dewar research group in this area was SAM1 (semi-*ab-initio* model 1) (90,91) in which two-electron integrals are calculated, rather than being parameterized. Enhancement with d orbitals has also been done and is commercially available in AMPAC (84). MNDO/d has d atomic orbitals added to the second-row atoms and gives improved results for molecules with second-row elements (92). The PM5 Hamiltonian is available in CAChe (Computer-Aided Chemistry, and now called Scigress Explorer), a commercial software product for computational chemistry (93) but has been minimally described in detail in the open scientific literature (94). Work on improving parameterization continues, resulting in incremental improvements in mean unsigned error of heats of formation (and other properties) of test compounds. Recent reports have been published on RM1 (Recife model 1) (94) and PM6 (95), both further refinements/improvements in parameterization. RM1 parameters are available electronically as supplemental material to the publication, and can be plugged directly into implementations of MOPAC in place of AM1 parameters. RM1 and PM6 have been implemented commercially (84).

Each of these methods moves the computational results toward greater overall agreement with selected experimental properties—primarily heat of formation, ionization potentials, dipole moments, and molecular geometries. However, inevitably with each of these parameterized methods there are some classes of compounds and some types of properties where there remain deficiencies or where some specific properties were actually handled better by an earlier generation of semiempirical theory than by a subsequent one. The methods also differ in coverage of elements. AM1 and RM1 are parameterized only to cover the “pharmaceutically relevant” elements—C, H, N, O, S, P and the halogens. PM3, PM5, and PM6 have sought to cover more elements of the periodic table.

Recently, William Jorgensen’s research group has published further refinements of the neglect of diatomic differential overlap (NDDO) formalism (upon which the AM1, PM3, SAM1, and other methods are based) by introducing a pairwise distance-directed Gaussian (PDDG) improvement (96,97,98). Further gains in improvement of mean unsigned error for compound test sets suggest that the semiempirical methods can be improved further to compete better with the widely used *ab initio* and composite methods.

Density Functional Theory

Another quantum mechanical implementation is density functional theory (DFT) (99,100,101,102). It is useful in both molecular and materials applications. DFT has the advantage of being roughly intermediate in speed and accuracy between semiempirical and *ab initio* calculations. Optimized geometries from DFT can be quite good. For these reasons, use of DFT by chemists has increased. The 1998 Nobel Prize in Chemistry recognized Walter Kohn and John Pople, proponents of DFT and *ab initio* theory, respectively (103).

Just as in solving the Schrödinger equation in Hartree–Fock theory, the total electronic energy in DFT is the sum of the kinetic energy of the electrons, the electron–electron repulsion energy, and the nuclear–electron attraction energy. Analogous to the Hartree–Fock equations, the Kohn–Sham equations are iteratively solved in DFT. A key difference is the appearance of a

so-called exchange-correlation functional, which adds some electron correlation and therefore helps improve accuracy. Functionals are functions of functions. Exchange-correlation functionals go by acronyms like B3LYP (Becke 3 term with Lee, Yang, and Parr exchange), B3PW91 (Perdue and Wang), and BP86. There are a large number of functionals available in the literature. B3LYP is one of the most widely used because of its superior accuracy for properties such as electron ionization energies and electron affinities. The de facto first-choice functional for calculations on organic molecules. B3LYP is a hybrid functional, being partially parameterized, and partially *ab initio*, and as such could also be considered as semiempirical.

Introducing Electron Correlation—Post-Hartree–Fock Methods

All of the approximations discussed thus far neglect electron correlation in one way or another—the ability of electrons to “instantaneously” respond to changes in the neighboring electrons’ positions. The post-Hartree–Fock methods attempt to correct this deficiency with more involved calculations, coming of course at substantially greater computational expense. Configuration interaction, coupled cluster theory, Møller–Plesset perturbation theory and complete active space SCF (CASSCF) are some of the approaches. Composite energy methods (also called multilevel methods) (104) such as G2(MP2) (Gaussian-2 Møller–Plesset second order) and its successors, empirically combine the results of a number of very high-level calculations on a molecule in order to obtain more accurate results—accurate meaning closer to real experimentally measurable values. The important point to remember is to keep in perspective the primary purpose of the computations. If absolute accuracy is required of the computed results, then the computationally expensive methods are necessary. If comparative modeling and trending is required, the much more expedient, albeit less accurate methods suffice.

SOFTWARE

For studying degradation pathways, one must investigate reaction mechanisms, which in turn must include an examination of the electronic structure of the atoms involved. A full palette of quantum mechanical methods should be at the heart of any software selected for use. The software package should include semiempirical MO methods (for treating large molecular systems and for preliminary reconnoitering of reaction pathways), *ab initio* MO methods with a variety of basis sets, and DFT implementations. The software should be bundled with molecular mechanics methods with reliable force fields for fast geometry optimizations and explorations of conformational space. All these methods should be accessible through an easy-to-use GUI that will allow for building the molecular structures on which the calculations are to be done. The GUI should present the program options (methods and parameters) in readily understandable menus. Most programs have a number of options available to the user pertaining to how a calculation is to be done. A well-designed program will have default options that perform well in many circumstances, so the user does not have to pick between obscure choices. The program should also easily export output for further analysis.

A catalog of computational chemistry software was published with brief descriptions of each software package, information about developers and vendors, and resources on the Internet (105). A somewhat more recent description of some relevant programs is given by Young (3). A variety of free software packages are available from individual developers, but availability depends on the whims and interests of the programmers. Today most of the computational chemistry software used in industrial, academic, and other laboratories is from commercial sources. Prices of these programs range from modest to expensive. Cost depends not only on the capabilities of the program, but also on the number of users at a site, the convenience of use, the level of technical support offered to users, and the number of competing software products. We list some of these companies in Table 1.

Most chemists are familiar with structure drawing programs, such as ChemDraw (106,107), MDL ISIS-Draw [or its successor Symyx Draw (108)], and ACD/ChemSketch (109). Although these programs do not rigorously enforce three-dimensional structure representation

Table 1 Some Commercial Suppliers of Computational Chemistry Software

Vendor	Website (www. *.com)	E-mail	Telephone	Headquarters
Accelrys	accelrys	solutions@accelrys.com	1-858-799-5000	San Diego, California
CAChe Group of Fujitsu	cacheoftware	info@cacheoftware.com	1-503-531-3600	Beaverton, Oregon
Chemical Computing Group	chemcomp	info@chemcomp.com	1-514-874-9538	Montreal, Canada
Gaussian, Inc.	gaussian	info@gaussian.com	1-412-279-6700	Pittsburgh, Pennsylvania
Hypercube Inc.	hyper	sales@hyper.com	1-352-371-7744	Gainesville, Florida
Parallel Quantum Solutions	pqs-chem	sales@pqs-chem.com	1-479-521-5118	Fayetteville, Arkansas
Planaria Software	arguslab	info@planaria-software.com	fax 1-206-440-3305	Seattle, Washington
Schrodinger, Inc.	schrodinger	info@schrodinger.com	1-503-299-1150	Portland, Oregon
Scientific Computing & Modelling	scm	info@scm.com	31-(0)20-444-7626	Amsterdam, The Netherlands
Semichem	semichem	sales@semichem.com	1-913-268-3271	Shawnee, Kansas
Tripos, Inc.	tripos	info@tripos.com	1-314-647-1099	St. Louis, Missouri
Wavefunction, Inc.	wavefun	sales@wavefun.com	1-949-955-2120	Irvine, California

rules, they do an admirable job of projecting such structure onto the plane of a piece of paper. These programs are sometimes better at rendering structures in a form easily understandable by all chemists than the GUIs of the computational chemistry packages. ChemDraw and similar programs [some of which are free (110)] can export structures in common molecular file formats that can be imported into computational package GUIs, and are therefore a convenient place to start to represent molecular structures. An excellent open-source molecular structure viewer, written in java, is Jmol (111). Jmol is capable of reading and displaying a large number of molfile formats, including output files from many of the major computational chemistry programs. New formats can be easily added to Jmol. Two other available molecular viewers are gOpenMol (112) and Molekel (113).

However, check structures closely before and after transfer between software packages to make sure that they are correct. Structure-representing assumptions may not be the same from package to package. For example, organic structure stick figures generally imply carbon-bound hydrogen atoms, which need to be explicitly specified for computational purposes. Two-dimensional molfile importation into one commercial GUI gets carried away and often over-decorates structures—particularly aromatic nitrogen atoms—with extra hydrogen atoms. Two-dimensional molfiles of modestly complex structures are not easy to translate into three-dimensional structures. Never trust the software to do what you want it to do until you have verified that fact.

Some programs are particularly potentially useful to the reader: SPARTAN (Wavefunction, Inc.), Jaguar (Schrodinger, Inc.), Gaussian/GaussView (Gaussian, Inc.), and AMPAC (SemiChem, Inc.). All run on a range of machines, from Unix-type workstations to window-based and Macintosh PCs. SPARTAN is a slightly more general molecular modeling program because it has molecular mechanics capability. ADF [Amsterdam Density Functional, scientific computing, and modeling (114)] specializes in DFT. All programs have strong and easy-to-use

quantum chemistry capabilities. Most have a GUI available. The largest vendor in the computational chemistry market is currently Accelrys, which has product offerings for chemistry, materials science, and the life sciences. Tripos, Inc. also vends a broad range of software products and services. The Chemical Computing Group (115) offering, called MOE (molecular operating environment), does molecular mechanics modeling exclusively, implementing the MMFF94 and other force fields, but provides interfaces to the quantum chemistry packages of other companies. It is particularly good at doing conformational searching, being efficient at finding globally optimal structures, which can then be further studied upon transfer to a quantum mechanics package.

Additionally, some quantum chemistry and associated molecular graphics programs can be obtained (licensed) at minimal or no cost. MOPAC is semiempirical and available from Stewart Computational Chemistry (116). GAMESS [General Atomic and Molecular Electronic Structure System (117)] can execute *ab initio* calculations on a variety of current computing platforms (118). The various versions of GAMESS are maintained at the Iowa State University and other academic laboratories. Another rather complete computational chemistry program is NWChem (119,120), and like GAMESS, it is available free. ORCA (121) from the University of Bonn is capable of DFT, *ab initio*, and some semiempirical calculations. It is particularly used for predicting spectroscopic properties of molecules—NMR chemical shifts and UV-vis spectroscopy.

Fifteen to twenty years ago, most computational chemistry software was designed to run on mainframe computers, minicomputers, and workstations. Such computers were the only devices capable of conducting such calculations in an effective and timely manner. More recently, with the increasing speed and declining price of computer chips, a desktop personal computer (PC) or a laptop computer has become an adequate platform for many calculations. The availability and price-effectiveness of PCs makes it practical to have a large number of small computers working on individual computations—or even clustered together for “distributed” computing—in order to accomplish large numbers of calculations and/or long calculations efficiently.

PRACTICUM

We have given an overview of the most used methods of computational chemistry. In this section, we show the practical steps the researcher might take to perform a typical computational experiment.

- If your workspace is at a large company or university, the software you need may already be installed somewhere at your institution. For someone starting from scratch, the first step would be to purchase or download free the computational chemistry software you plan to use. We have already mentioned some of the popular programs. The Google search engine will help you locate software and vendors. Nowadays, the commercial software can be purchased from the vendor's website. Some vendors will let the purchaser download the product over the Internet, whereas others will mail a compact disc along with installation instructions. If you are in private industry, the price will be significantly higher than if you are not. Once installed, the software can usually be used until you have to buy another computer, in which case the new machine will likely have a new operating system, which will necessitate buying the latest version of the software.
- Once the software is running, the next step is to enter the molecule(s) you want to treat into the computer. This is easy to do with software that has a GUI. In such a case, entering a molecule is similar to drawing a chemical diagram such as with ChemDraw. The difference is that a molecule in ChemDraw is two dimensional, whereas nonplanar molecules are treated as true three-dimensional objects in computational chemistry methods. Consult the user manual for the software for details about building molecular structures.

- Hydrogens matter in computational chemistry. Chemists are used to shorthand notation of leaving off the hydrogen atoms in chemical diagrams. This will not work for molecular mechanical or quantum mechanical treatments. Hence, when a structure is assembled on a computer screen, the user must make certain that the valences of each nonhydrogenic atom are appropriately filled with hydrogens. Also, ionic species must be built appropriately. Thus, a carboxylate group consists of -COO^- ; an ammonium group should be -NH_4^+ . Charge on the molecule is also important. For quantum mechanical treatments, if the molecule is an anion, it should be given an integer negative charge. If the structure is cationic, it should be assigned an integer positive charge. Zwitterions are given a net zero charge, but the presence or absence of the hydrogens will tell the program which are the charged groups. The molecular charges are used by quantum chemistry programs to figure out how many electrons should be in the structure. Treating radicals requires special consideration (122). As an aside, something to realize in calculating the energy of dissociation of a proton from a molecule is that the quantum mechanical electronic energy of a proton is zero. This is so because the proton has no electron.
- It is considered standard practice to optimize the molecular structures before further study. In other words, minimize the energy of each molecule to be studied. This structure optimization based on energy should be done by molecular mechanics with a good force field (e.g., MMFF) or quantum mechanically. A commonly used strategy is to optimize the structure with a modest basis set (e.g., one labeled 6-31+G* or something similar; see any of the recent computational chemistry textbooks for an explanation of this obtuse labeling system). Once optimized, higher-level (e.g., with larger basis sets) calculations can be done to address your particular research questions. A quantum mechanical calculation without geometry optimization is called a single point calculation because the results describe just one configuration of nuclei on the potential energy surface.
- Bear in mind that the molecule(s) should be put in a suitable conformation or multiple conformations if that is germane to your research question. If a reaction mechanism is being studied, put the reactants and products in appropriate configurations similar to how you would envision the molecules coming together in space or in solution. So as to not prejudice the results, several possible mechanisms and configurations should be modeled. Computational results are best used for comparison among themselves. In other words, trends and relative predicted values for a series of similar compounds are more meaningful than absolute values. If intramolecular hydrogen bonding exists in the molecule being treated, the H-bonded hydrogen should be pointed in the direction of the electron pair donor. It is a good practice to reoptimize a molecule after its conformation is tinkered with. In fact, it does no harm and does not take much computer time to optimize a structure twice in a row to make sure that it is at an energy minimum.
- In modeling reaction mechanisms, it is sometimes the goal of the researcher to determine the transition state structure. This can be found by approaching the saddle point on the potential energy surface from both the reaction side and product side. Many quantum chemistry programs have the ability to compute vibrational frequencies. If one and only one of the resulting vibrational frequencies is negative (usually in the range -50 to -300 cm^{-1}), this signifies your calculations have located a transition state. If two vibrational frequencies are negative, the structure is at a peak on the potential energy surface.
- Much of quantum chemistry is done on isolated molecular structures, and therefore corresponds to molecules in a vacuum. This obviously gives only a rough idea of what happens in crystalline material, in the liquid phase or in solution. If the software you are using has an option to include a solvent model along with a quantum mechanical calculation, it is worth trying and may give results more relevant to your actual experiments (see comments below on solvation modeling). The solvent models in quantum chemistry programs operate on the concept of trying to mimic the environment produced by a solvation shell rather than treating the solvent molecules as explicit entities.

APPLICATIONS

Conformational Analysis and Atropisomerism

Addressing conformational questions is a strong suit of computational chemistry and molecular modeling. These computations are almost always done on the ground state, closed-shell (i.e., all electrons paired) systems, which the computational procedures are generally designed to model. Both quantum mechanics and molecular mechanics work well at predicting relative conformational energies. Quantum mechanics is very reliable at determining barriers to internal rotation. (Molecular mechanics can sometimes give good barriers, although it is primarily parameterized to reproduce the energies and geometries of molecules in their equilibrium states.)

A simple and classic example is the dependence of total energy on the central dihedral (torsional) angle of *n*-butane (123). Calculated energy as a function of dihedral angle for *n*-butane is shown in Figure 2. The global energy minimum corresponds to the *trans* conformation, indicated by the associated Newman projection in Figure 2. The global energy maximum corresponds to the conformation where the two terminal methyl groups are eclipsed (but shown slightly staggered in Fig. 2 for clarity). The other Newman projections mark the remaining eclipsed (local energy maxima) and staggered (local energy minima) conformations. Note that the energy difference (on the *y*-axis) between the global maximum and minimum is only slightly larger than 3kcal/mole, entirely consistent with normally expected "free" rotation about the central carbon-carbon bond.

An effective use of this kind of computational modeling comes when the calculations can predict that an energetic barrier to free rotation is large, such that conformations cannot readily interchange. The phenomenon of atropisomerism starts to appear. High-performance liquid chromatography can sometimes separate atropisomers of a compound with highly hindered rotation about a single bond, producing effectively two molecular species. The classically cited example of atropisomerism is the two isolatable forms of 6,6'-dinitro-2,2'-diphenic acid (structure 3) (124,125).

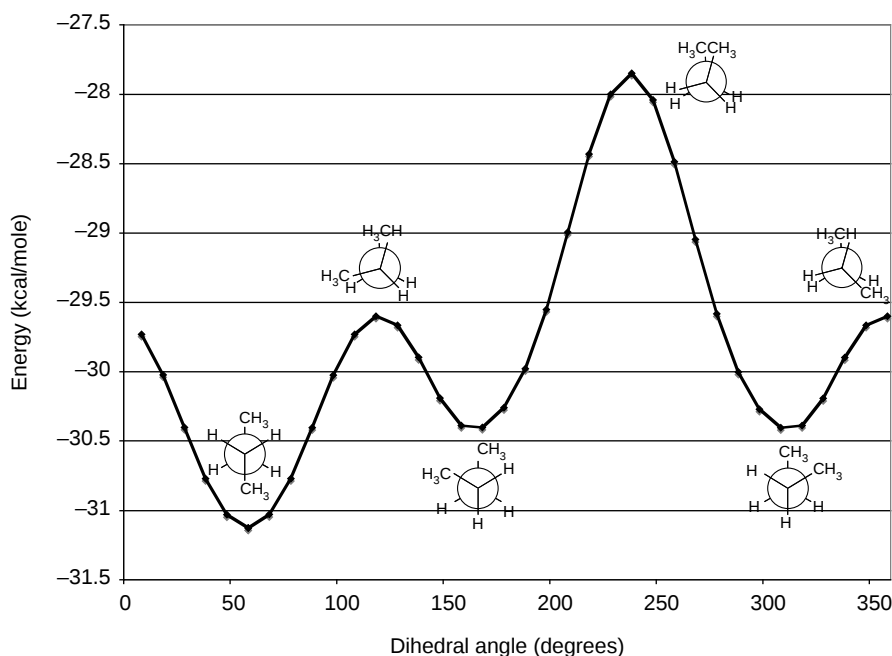


Figure 2 Conformational energy as a function of central dihedral angle for *n*-butane. Calculated using semi-empirical model AM1, implemented in AMPAC.

Computational modeling of this compound reveals several aspects, both about the chemistry and about computational limitations. Calculations were done on structure 3, as well as on biphenyl (structure 1) and 2,2'-diphenic acid (structure 2) for comparison; these are shown in Figure 4.

The top panel of Figure 4 is a vertical expansion of the conformational energies calculated for rotation of biphenyl around the central carbon–carbon bond connecting the two aromatic

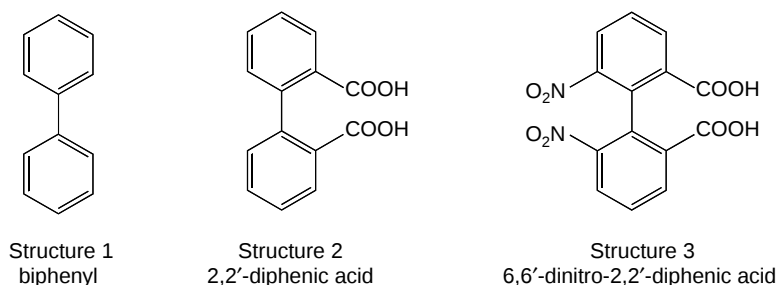


Figure 3 Chemical diagrams of biphenyl, 2,2'-diphenic acid, and 6,6'-dinitro-2,2'-diphenic acid.

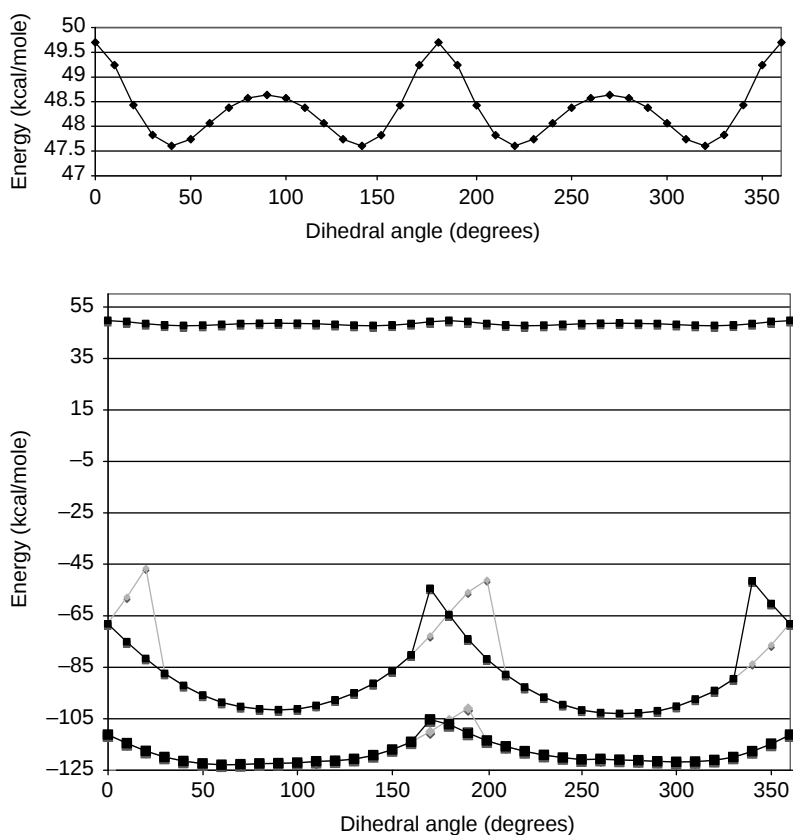


Figure 4 Computed conformational energies as a function of central dihedral angle for biphenyl (upper panel and upper curve of lower panel), 2,2'-diphenic acid (lower curves of lower panel) and 6,6'-dinitro-2,2'-diphenic acid (middle curves of lower panel). The energy (heat of formation) was calculated using semiempirical model AM1, implemented in the AMPAC program. Note the different energy scales in the upper and lower panels.

rings. The four energy minima occurring at 47.6 kcal/mole correspond to the four conformations where the two planar aromatic rings form a 40° angle with respect to each other. The two energy maxima at 49.7 kcal/mole correspond to both rings lying in the same plane. The two intermediate energy maxima at 48.6 kcal/mole correspond to a dihedral angle of 90°. The difference between global energy maximum and minimum is only 2.1 kcal/mole.

This same conformational energy profile for biphenyl is plotted again in the lower panel as the upper nearly flat line at approximately 48 kcal/mole, this time on the same *y*-axis scale as similar calculations done for 6,6'-dinitro-2,2'-diphenic acid (middle set of curves) and 2,2'-diphenic acid (lower set of curves) for comparison. Notice the different magnitudes of heat of formation for the three compounds. The larger compounds have a more negative heat of formation. The energies plotted are theoretically computed heats of formation obtained by AM1 semiempirical MO calculations. The programs MOPAC and AMPAC are parameterized so that the heats of formation they predict should parallel actual thermodynamic heats of formation. Also notice the differences between the maxima and the minima conformational energies. We plot profiles for these compounds on the same energy scale to emphasize the differences—something that is easily missed if the relative energies were graphed in three separate plots.

Next, we note the dramatic discontinuities in the profiles for diphenic acid and dinitrodiphenic acid. These are not features of the chemistry, but rather eccentricities of the calculations. The two pairs of profiles result from computationally rotating the central dihedral angle in the positive (e.g., clockwise) direction and in the negative (e.g., counterclockwise) direction. In the case of diphenic acid (lower energy profiles), the dihedral angle corresponding to approximately 180° is the conformation in which the two carboxylic acid groups approach each other, interfere with each other spatially because of the physical size of the groups, and do not permit internal rotation to continue. The mathematics used to model such conformations does not care that it is modeling an “impossible” conformation. A well-built, rugged computational algorithm will find the best solution—that is, the conformation satisfying the mathematics—disregarding chemical reasonableness. The 160° and 200° dihedral angles correspond approximately to where the two carboxyl groups come into van der Waals contact with each other. Although rotation is hindered in this direction, rotation in the opposite direction allows free access to all reasonable conformations with an approximate 15 kcal/mole energy barrier. This compound would therefore not exhibit atropisomerism.

Not so with dinitrodiphenic acid (structure 3). The bulky nitro and carboxyl groups on positions 6, 6', 2 and 2' of the aromatic rings physically restrict this molecule to two chemically isolatable conformations, although no chiral center exists in the molecule. As with diphenic acid, the calculations ignore the physical impossibility of the bulky groups rotating past each other. The discontinuities in the curves, depending upon whether the central bond is rotated positively or negatively, indicate the situation. The onsets of the discontinuities reflect the dihedral angles where van der Waals contact is made. The black and gray curves distinguish whether the central dihedral angle variation is driven clockwise or counterclockwise. Once the bulky side groups are forced past each other, the molecular energies settle to a smooth curve.

The cases above are dramatic and do not significantly challenge a good organic chemist's structural intuition. However, the case of “ring flips” can be trickier. The ring flip from chair to boat conformation in cyclohexane is modeled in Figure 5. The dihedral angle is driven in the calculations from -70° to +80° in one series of calculations, and in the opposite direction in another. The global energy minimum conformation corresponds to the chair conformation. The barrier, by these calculations, is approximately 5 kcal/mole. Driving the calculation in reverse indicates the additional feature that the strict boat conformation is not the lowest energy conformation—rather the two twist-boat conformations on either side are of slightly lower energy. If absolutely necessary, these abrupt discontinuities in the curves can be likened to the phenomenon one experiences when assembling a ball-and-stick model of cyclohexane, then physically twisting the ring until it pops from one conformation to the other.

How is such modeling useful, when the results in the extreme case of the substituted diphenic acids are obvious? In more subtle cases, where restricted rotation or restricted

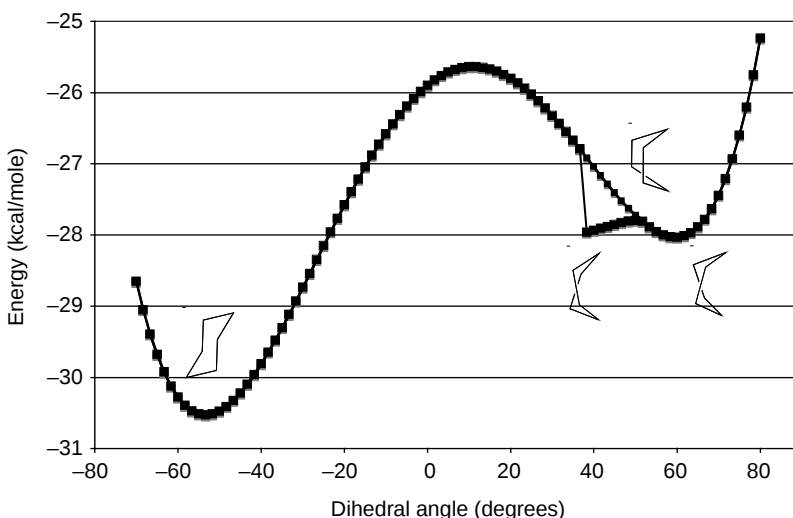


Figure 5 Computed conformational energy as a function of dihedral angle to cause ring flipping of cyclohexane. Calculated using semiempirical model SAM1/d implemented in AMPAC.

conformational switching is suspected, computation of energetic barriers is useful to see if the possibility exists, or is unreasonable. Clearly, if steric hindrance does not preclude free rotation, then the height of energy barriers will influence the lifetime of conformations. Temperature dependence will be expected as Eliel and Wilen (123) review. While atropisomeric differences may not be dramatic enough to permit “isolation” of isomers, the phenomenon may be detectable by such as NMR or other spectral study, or by HPLC retention time, as mentioned in the beginning of this discussion.

Solvation Modeling

The majority of computational chemistry calculations are done on isolated molecules, usually in the gas phase. Because pharmaceutically active molecules exist and have their effect in the condensed phase (in solution), the relevance of gas phase calculated results comes into question. Solvation models have been developed to simulate bulk solvation effects and solvation shells. Such calculations are available in several of the computational chemistry software packages. The Klamt COSMO (conductor-like screening) (126,127) model calculates dielectric screening effects in solvents. The COSMO model is available in numerous of the computational chemistry software packages (93,121) and both COSMO and the Cramer–Truhlar solvent model (128) are available in the AMPAC software package (84). Spartan has two solvation models available: SM5.4 (129) and SM8 (130).

Modeling Hydrolysis

Hydrolysis is one of the most common reactions encountered. Degradation of a pharmaceutical agent can occur if it is stored in the aqueous solution or if it is exposed in the solid state to any water vapor during storage, formulation, distribution of the dosage forms, or patient handling. Hydrolysis can be especially problematic for susceptible compounds that are hygroscopic in the solid state.

Hydrolysis reactions have often been modeled with computational chemistry. You can start the computational experiment by modeling the substrate (drug) molecule. Then a nucleophile can be “flown” toward the site of attack, for instance, the carbonyl carbon of an ester or amide linkage (131). The model nucleophile can be a water molecule or a hydroxide ion.

A typical computational experiment would be to calculate the quantum mechanical total energy of the substrate and nucleophile fixed at a separation of 4 Å. Then incrementally move the nucleophile closer in steps of 0.25 Å until the reactants are within about 1.25 Å of each other. The energy of the system is computed at each step. With gas-phase modeling, the energy will go down until the separation is close to that of a covalent C–O single bond length (i.e., ca. 1.4 Å), and then at still closer distances the energy will start to rise rapidly indicating less stability when the reactants are pushed too close.

The change in total energy between the infinitely separated reactants and the species at the energy minimum of the reaction coordinate curve will not be comparable to the heat of reaction (because thermodynamics is neglected in the quantum mechanical model). Nevertheless, the energy change can be quite useful on a relative basis in the case of comparing the hydrolysis of a series of closely related compounds.

Let us give a specific example. Cephalosporins are well-known antibacterial agents. There are tens of cephalosporins on the market and thousands more in the patent literature and in proprietary company archives. A common route of cephalosporin decomposition is via hydrolysis of the β -lactam ring and subsequent rearrangements. The biological mode of action is for the β -lactam ring to open when acylating the hydroxyl group of a serine in the active site of bacterial transpeptidases (132). In effect, the nucleophile ($-\text{OH}$) reacts at the carbonyl carbon of the β -lactam.

One of the sites of substitution differentiating cephalosporins is position 3 (Figure 6). Modeling experiments were designed (133) to compare the energy profile for a hydroxide ion attacking the β -lactam carbonyl of a simplified 3-cephem ring system with various substituents at R_3 . It was found that the energy change along the reaction pathway correlated with Gram-negative antibacterial potency of the compounds (134). Moreover, these calculated energy changes correlated with experimental base-catalyzed hydrolysis rates, chemical shifts at the Δ^3 double bond, and carbonyl stretching frequencies (135,136). The result was a tidy picture where computed properties, physicochemical properties, kinetics, and biological activities tied together in a rational and coherent way. It was not necessary to compute true heats of formation because the modeling experiments were designed so that relative energies (corresponding to the different R_3) could provide useful answers. In fact, a semiempirical MO method sufficed. The key point is that the computational experiments were set up so that systematic errors would roughly cancel, and results were obtained that could be compared.

A recent report suggests that the Fukui frontier molecular orbital (FMO) approach may be useful in identifying likely hydrolysis sites (229).

Open-shell Molecules

By far, most calculations reported in the chemical literature are on closed-shell molecules, that is, the singlet state, those with the molecular orbitals occupied by two electrons each, one of α spin and one of β spin. However, in the case of radicals (the doublet state) there is a singly

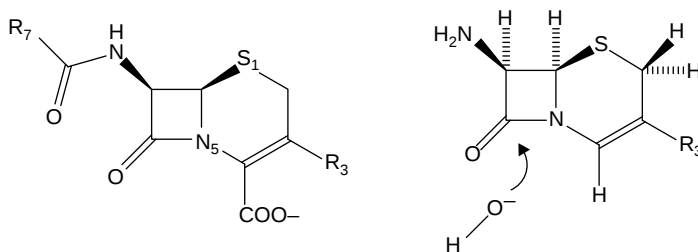


Figure 6 Chemical diagrams of the general structure of cephalosporin antibacterial agents (*left*) and the model structure (*right*) used in calculations to determine the electronic effect of R_3 on the geometry and reactivity of the β -lactam ring. The hydroxyl anion was moved perpendicularly toward the carbonyl carbon of the β -lactam ring from below. The 3-cephem ring system is numbered clockwise around the six-membered ring and then counter-clockwise around the four-membered ring.

occupied orbital, or in the case of diradicals (triplet state) two singly occupied orbitals. These radicals are referred to as open-shell molecules. Calculations on these are more challenging than those on closed-shell molecules as explained in the next two paragraphs.

One way to deal with unpaired electrons is to use the unrestricted Hartree–Fock (UHF) treatment. Whereas regular *ab initio* calculations restrict the one-electron spatial orbitals to be identical for α - and β -spin electrons (the restricted Hartree–Fock treatment, RHF), in UHF the spatial orbitals are allowed to be different in the SCF computation. Usually, the difference in the spatial orbitals for α and β electrons is only slight. Unfortunately, when applied to a radical, UHF stumbles into a pitfall (137) called “spin contamination.” UHF wave functions cannot be trusted to correspond to pure spin states such as a doublet for radicals or a singlet or triplet for diradicals. Theoretically speaking, the UHF wave function may not be an eigenfunction of the spin operators.

Another way to computationally treat unpaired electrons is to employ restricted open-shell Hartree–Fock (ROHF) theory. Here we encounter another pitfall. It is an artifact called “symmetry breaking” (137). Whereas ROHF wave functions are pure spin states, the ROHF wave function may not retain the symmetry of the molecule. Suppose a molecule has C_{2v} symmetry. The wave function should have the same symmetry, for example, the orbitals lobes on either side of the symmetry plane should be identical. However, with symmetry breaking, the two sides are not equal. The unsymmetrical ROHF wave function may even give lower energy than a physically correct (symmetrical) wave function. Symmetry breaking occurs because correlation between electrons of opposite spin is insufficient.

What is one left to do? It has been recommended (137) that the most reliable method for treating radicals in general is DFT. With a functional such as B3LYP, unrestricted DFT gives good energies and optimized geometries. Time-dependent DFT (TDDFT) is a method seeing increased usage because its results are easy to interpret (114).

Carbon–Hydrogen Bond Dissociation Energies

With the commentary about calculations on open-shell molecules in mind, we nevertheless consider making use of such calculations here. Hydrogen atoms on an organic molecule that can be easily removed are potential sites of oxidation. Although there are several different kinds of oxidation mechanisms, they share the one general step that, typically, a hydrogen atom has to be removed from the molecule. How easy this removal can be achieved thermodynamically determines the propensity of the site on the molecule toward oxidation. The thermodynamic expression for a hydrogen bond dissociation enthalpy is given as follows where the superscript dot signifies, as usual, a radical:

$$D_{RH} = \Delta H_f[R^\bullet] + \Delta H_f[H^\bullet] - \Delta H_f[RH]$$

D_{RH} is a measure of the energy required to remove a hydrogen radical from its parent molecule, leaving behind a radical. It is presumed to be a reasonable approximation to an intermediate on the way to oxidation of the molecule. Computationally, modeling this behavior opens up the problems referenced earlier for calculations of open-shell chemical species.

The terms “bond dissociation energy” and “bond dissociation enthalpy” are sometimes used interchangeably. Actually, the dissociation energy refers to the energy required to break a bond at 0°K. Dissociation enthalpy is adjusted to a particular prevailing temperature, typical 298°K. The semantic difference between the terms can be considered innocuous as the values are almost numerically equivalent for most organic molecules (138). Experimental values differ by 3 kcal/mole or less on average. The average unsigned error reported for the PM6 semiempirical method (139) is comparable at 4.4 kcal/mole.

Bond dissociation energy, conceptually, is differentiated from bond energy. March (140) discusses the origins of commonly cited bond energy values for organic molecules. For instance, the C–H bond in simple organic molecules is in the range of 96–99 kcal/mole. These numbers derive from total combustion experiments in which, for example, the C–H bond energy for

methane is the combustion energy, 397 kcal/mole (at 298 K), divided by 4 (the 4 C–H bonds in methane). Values derived by combustion of progressively larger hydrocarbons decrease a small amount, to give the 96 to 100 kcal/mole range.

Sequential removal of a hydrogen atom from methane, however, results in (experimentally measured) bond dissociation energies (BDEs) of 105, 110, 101, and 81 kcal/mole, respectively (138). Although these values add up to the 397 kcal/mole (at 298 K) value for total combustion, the bond dissociation of each hydrogen is different. The energy required to break an individual bond is dependent on the rest of the structure to which it is attached. For this reason, specific bond dissociation energies, dependent upon an associated structure, are more appropriate indicators of specific carbon–hydrogen bond strengths, and of oxidative susceptibility at a particular site on a molecule.

Sequentially removing a hydrogen atom and determining the carbon–hydrogen bond dissociation energy for each hydrogen environment on an organic molecule is an appropriate computational exercise. A set of high quality experimental measurements on 39 compounds (13 primary compounds and 26 additional compounds) was available from the literature (141) and used as a calibration set for such an exercise. The results of calculations done using two models, the semiempirical SAM-1 UHF model and a DFT UHF model using the B3LYP functional and the 6-31G basis set, are shown in Figure 7. A full report of this study is in press (230). Kieffer et al. (229) propose this same evaluation, but without significant validation, and at greater computational expense.

A substantial amount of scatter is evident in Figure 7, left panel (computed using the SAM-1 semiempirical method). What is the source of this scatter? The value for $\Delta E_{\text{formation}}$ for $\text{H}^\bullet$ used in the bond dissociation energy equation is a constant. Statistics on the accuracy of $\Delta E_{\text{formation}}$ calculations for the closed-shell reference compounds have been published in the literature and are consistent with the calculations here. Does the scatter simply reflect the imprecision of the semiempirical methods? This imprecision is implicated by comparison with results obtained when the calculations were repeated using DFT UHF B3LYP/6-31G (Fig. 7, right panel), at appropriately higher computational cost. Scatter still exists, but the correlation is better. The B3LYP calculations overestimate BDE, whereas the semiempirical calculations underestimate. We assume that a primary source of scatter in the computed BDE values must be in

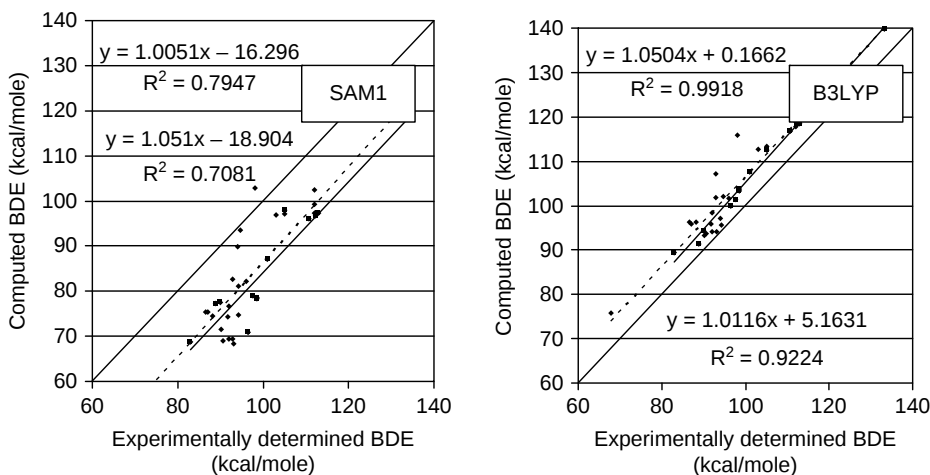


Figure 7 Correlation of experimentally measured bond disassociation energy (BDE) values with computed values. Left panel: calculation using the SAM1 semiempirical method. Right panel: DFT UHF B3LYP/6-31G calculations. In both panels, the diagonal solid line represents a perfect correlation. The solid lines fitted through the data and the upper regression equations are for the 13 primary reference compounds. The dotted lines fitted through the data and the lower regression equations are for the entire 26 compound reference compound set.

the computations of the open-shell species. The scatter suggests caution in comparing computed BDE values between molecules. However, comparing computed BDE values of different C–H environments within a molecule is still possible. Systematic errors would be expected to cancel in comparing positions within a molecule.

We have applied this BDE concept to molecules with which we have experimental experience, where degradation chemistry has been well characterized. Sertraline (structure in Fig. 8), as the crystalline hydrochloride salt, shows excellent stability empirically. As the free base or in solution, the compound shows measurable oxidative instability. The hydrogen atoms expected by an experienced organic chemist to be trouble spots on the molecule are the hydrogen atoms on carbons adjacent to the heteroatom (positions 1 and 9), and the double benzylic hydrogen (position 4).

Sertraline is manufactured as the hydrochloride salt to stabilize the molecule, consistent with the general principle that amine salts are oxidatively more stable than their free base counterparts. Comparable computations were performed on a protonated form of sertraline. Focusing on the low BDE values computed for the free base, the energies for hydrogen 1 and hydrogen 9 increased by approximately 10 kcal/mole between the free base form of sertraline and the protonated form. The increase in BDE for hydrogen 1 and hydrogen 9 reflects a reduction in the oxidative susceptibility of these hydrogens, consistent with the fact that sertraline hydrochloride is more stable than the free base, and consistent with general organic chemical principles. The BDE for hydrogen 4, however, remains relatively unchanged. These energy calculations support the facts that the hydrochloride salt is stabilized toward imine oxidation, but salt formation does not influence oxidative susceptibility at position 4.

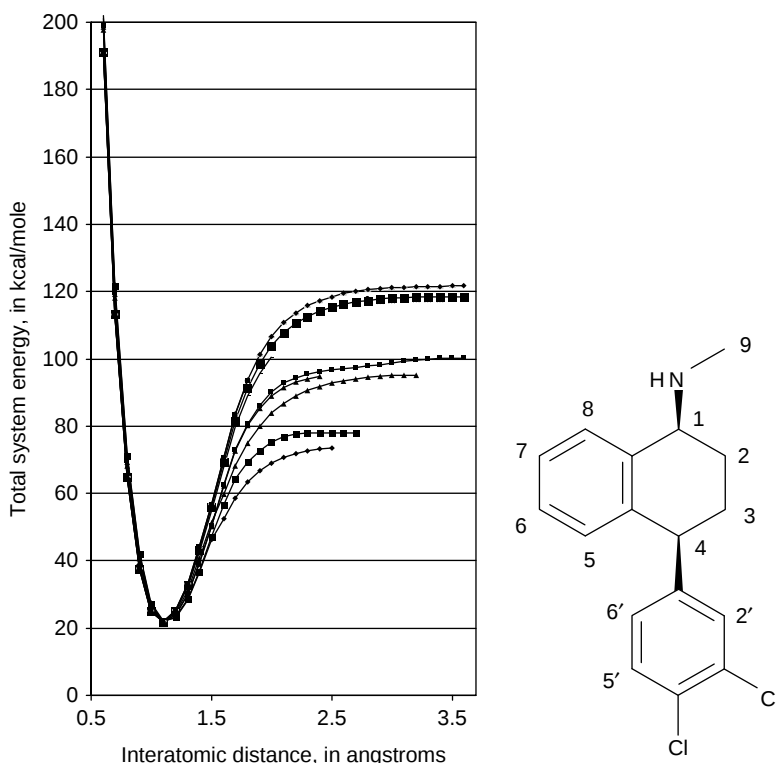


Figure 8 Computed energy of formation as a function of carbon-hydrogen interatomic distance for the 12 different hydrogen atom environments of sertraline ($C_{17}H_{17}NCl_2$ relative molecular mass 305 daltons monoisotopic). Values were computed using the SAM1 semi-empirical model, an unrestricted Hartree–Fock wave function, and no solvation model.

Theoretically computed bond association energies have been reported for a variety of systems (142,143). Recently, BDEs for an extensive set of hydroquinones and catechols were published (144). Conformations of the molecules were optimized by DFT at the B3LYP/6-31+G* level. The conformers of lowest energy were then used to compute BDEs by determining single-point total energies using DFT with the B3LYP functional and a large basis set, 6-311++G(2df,p). The energies were corrected for zero-point energies and thermal effects. Solvation was accounted for using a polarized continuum model of DMSO. Predicted values of one-electron oxidation potentials, pKa values, and first and second BDEs of hydrogen from these molecules were claimed to be in reasonable agreement with available experimental data.

Predicting UV-Visible Spectra

Electronic transitions in the UV-visible region are commonly used to detect compounds eluting from a chromatographic system. Computational chemistry provides a way to estimate transitions in UV-visible spectra of compounds even before they are isolated and characterized. An electronic transition involves excitation of an electron from the ground state wave function to one of the excited state wave functions. An adiabatic excitation is one that involves adjustment of the nuclear geometry to minimize the energy of the excited molecular system. A vertical excitation is one that occurs so rapidly that the ground state geometry does not have time to change. This latter type of excitation is usually adequate for modeling UV-visible spectra.

There are various post-Hartree-Fock methods for obtaining a wave function for an excited state (145). However, a molecule has so many excited states it may be computationally difficult to determine the wave functions for each of these except when symmetry (i.e., the irreducible representation in group theory) can be used to distinguish the states. For drug-sized molecules, an MO calculation is more practical than post-Hartree-Fock methods. An MO calculation will give a set of filled molecular orbitals and a set of unoccupied (virtual) molecular orbitals (and, in the case of radicals, one or more singly occupied molecular orbitals). Simplistically, electronic excitations can be conceived as promoting an electron from an occupied MO to an unoccupied one. In reality, of course, the molecular orbitals would adjust to the excitation so that the ground-state molecular orbitals are only an approximation to the excited state molecular orbitals.

A fast approach to predicting spectra is to use a semiempirical MO method specifically parameterized to reproduce experimentally observed electronic transitions. Such a method is INDO/S (intermediate neglect of differential overlap/spectroscopy). The most widely used INDO/S parameterization is that developed by Zerner and coworkers (81). The name of both the parameterization and the program is ZINDO. This semiempirical method does well for predicting spectra of organometallic compounds and organic dyes, such as used for photographic film or hair coloring. However, ZINDO is not good for predicting bond lengths and angles, so it is best to obtain a good geometry by molecular mechanics or ab initio calculations and then use this geometry for spectral calculations with ZINDO.

Computing an electronic excitation will give $\lambda_{\max}$ for the various predicted transitions. Determining the intensity of each peak in a spectrum involves calculation of transition dipole moments. Unfortunately, not all molecular modeling programs have facility to compute these. Relevant software products include the semiempirical MO programs ZINDO from Accelrys, CAChe, and MOPAC from Cache Software/Fujitsu, MOPAC from Schrödinger, AMPAC from SemiChem, ArgusLab from Planaria, Gaussian from Gaussian Inc., ORCA from the University of Bonn, and HyperChem from Hypercube. Many ab initio programs can also compute transition dipole moments. To broaden the sharp, calculated excitation lines into the peaks typically seen in an experimental UV spectrum, each line can be replaced by a Gaussian distribution curve and then these curves can be summed.

The UV-visible spectrum observed for a solution in a cuvette is a weighted average of the UV-visible spectra of all chemical species in solution. If a molecule has an ionizable group in

proximity with the chromophore, the molecule will likely exhibit a pH dependence of its UV-visible spectrum. pH-dependent changes in spectra are readily observable, and demonstrate the weighted average concept. Computing and comparing the UV-visible spectra for a free acid and its conjugate base (deprotonated) form, or an amine and its protonated form, is possible.

The UV-visible spectral properties of *p*-nitrophenol are described here as an example. The phenolic hydroxyl is weakly acidic, and has a measurable pK_a . It acts as a pH indicator—the pK_a is given in the literature as 7.3 (Fig. 9) (146). A dramatic UV-visible spectral shift occurs upon ionizing to the phenolate anion. The observed $\lambda_{\max}$ and extinction coefficient for *p*-nitrophenol free acid (in acidic pH) are 317 nm and $\epsilon = 0.9 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. The observed $\lambda_{\max}$ and extinction coefficient for *p*-nitrophenolate anion (in basic pH) are 402 nm and $\epsilon = 1.9 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. The isosbestic point is 345 nm. Observed spectra under acidic and basic conditions are shown in Figure 10. Semiempirical calculations by the PM5 parameterization give $\lambda_{\max}$ and extinction coefficient for *p*-nitrophenol free acid (in acidic pH) of 311 nm and $\epsilon = 3.2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. The computed $\lambda_{\max}$ and extinction coefficient for *p*-nitrophenolate anion (in basic pH) are 399 nm and $\epsilon = 6.2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. The isosbestic point is 347 nm (Fig. 11). The PM5 results which are for the gas phase, do indeed mimic the experimental observations. The point to remember from these results is that the ionization state of the molecule should be considered when comparing calculations and experimental results. pH dependence of the UV-visible spectrum should also be considered.

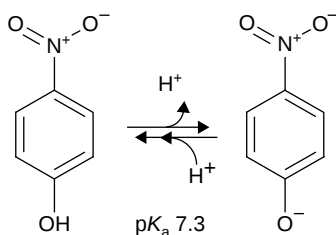


Figure 9 Ionization of *p*-nitrophenol.

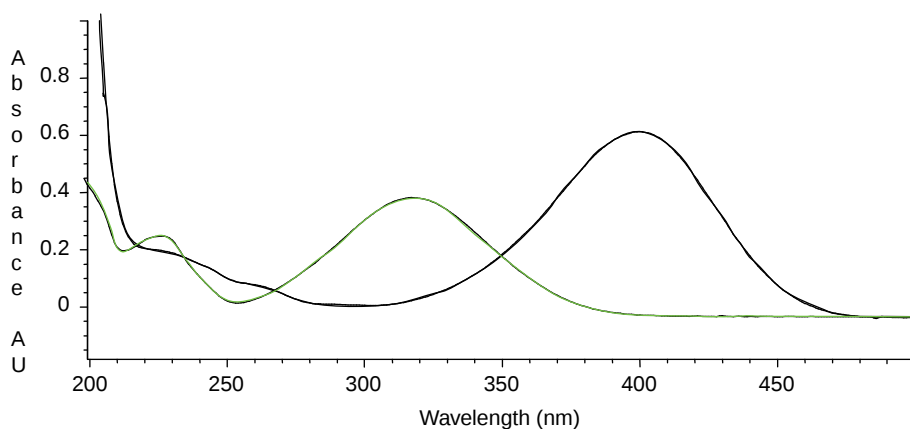


Figure 10 Observed UV-visible spectra of *p*-nitrophenol ($\lambda_{\max} = 402$) and *p*-nitrophenolate anion. The spectrum for the former compound is shown by the black curve; the spectrum for the anion is shown by the green (gray) curve. UV-visible spectral observations were made on a Hewlett-Packard Model 8453 UV-Visible spectrophotometer. Absorbance is plotted on the vertical scale; the values range from 0.0 to 0.8. Wavelength is plotted on the abscissa; values range from 200 to 450 nm.

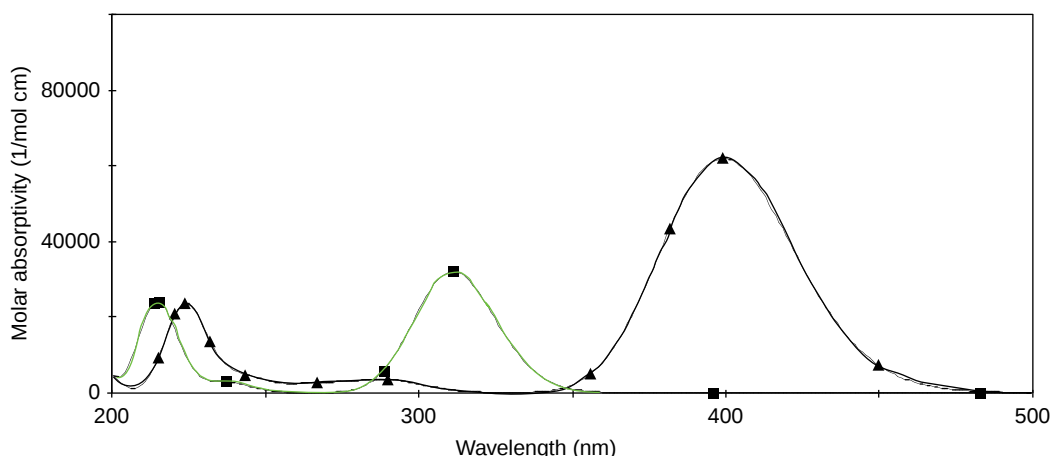


Figure 11 Computed UV-visible spectra of *p*-nitrophenol and *p*-nitrophenolate anion. The spectrum for the former compound is shown by the black curve; the spectrum for the anion is shown by the green (gray) curve. Computations were done using the CAChe molecular modeling system (Fujitsu of America). Specifically, molecular geometries were optimized using the semiempirical MO procedure with PM5 parameterization and including the COSMO model for water. Molar absorptivity is plotted on the vertical scale; the values range from 0 to 80000. Wavelength is plotted on the abscissa; values range from 200 to 500 nm.

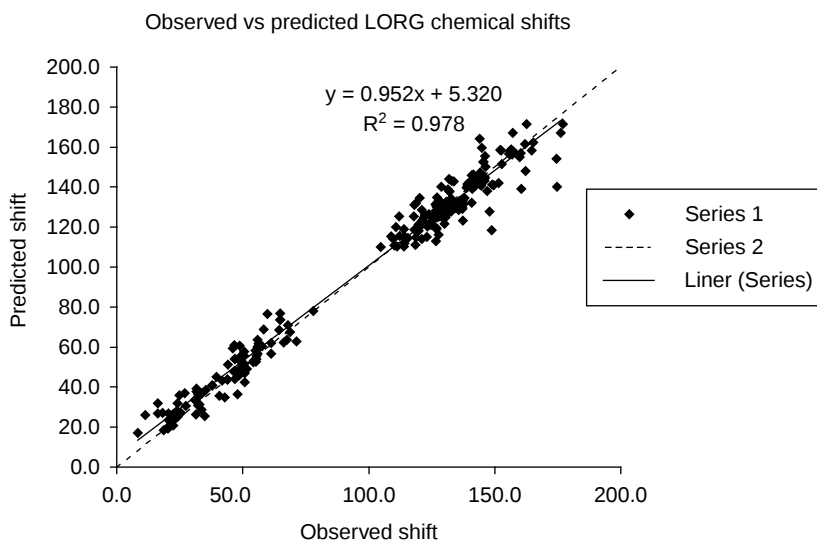


Figure 12 Computed versus observed ^{13}C chemical shifts of a composite of chemical shifts of 12 pharmaceutical molecules. DFT calculations using B88P91 and a small basis set.

NMR Chemical Shifts

NMR chemical shifts are determined by the electronic environment of atoms in a molecule. As such they should be computable with good electronic structure models. Cheeseman et al. have published results of comparisons, as have others (147,148). A comparison of ^{13}C NMR shifts of a small set of pharmaceutically relevant molecules, calculated by DFT with the B88P91 functional and a small basis set, are shown in Figure 12.

Vibrational Circular Dichroism

As chirality has become an important aspect of pharmaceutical development, establishing the absolute configuration of chiral centers is important in characterizing candidate drug molecules. The stereochemical fidelity of some synthetic reactions can sometimes be called into doubt. Ways to measure stereochemical configurations are therefore valuable. X-ray crystallography is the definitive method, but not always possible. Instrumental developments in the area of vibrational circular dichroism (VCD) – measuring the interaction of molecules with circularly polarized infrared radiation analogous to UV-visible circular dichroism – facilitates making such measurements. Interpretation of VCD spectra for obtaining absolute configuration, however, is not entirely possible without computational chemistry modeling. Minick et al. (149) summarize their experiences to date. Density functional calculations using the B3LYP functional and a high level (i.e., large) basis set are necessary. The calculations are not fast.

Photochemistry

As degradation chemists are well aware, one of the potential sources of degradative forces on pharmaceutical products is light. Pharmaceutical companies and pharmacists have long known that some biomedical products must be stored in dark bottles. Even bottled soda pop ages faster in the sunlight. Modeling photochemistry is especially challenging because besides the difficult task of finding the minimum energy pathways from the reactants to the transition state to the various products that can result from degradation, one must also deal with electronic transitions and hence pathways on more than one potential energy surface. The Gordian knot of modeling a photochemical reaction is the funnel region where an excited state reactant or intermediate descends to the ground state potential energy surface. The funnel region is called a conical intersection (150). In practical terms, a post-Hartree–Fock method (145) that is able to reliably optimize ground and excited states must be used. One such method is complete active space SCF (CASSCF), which is available in *ab initio* programs such as Gaussian. The drawback of modeling with a post-Hartree–Fock method is that the computer time requirements are heavy. Molecules with 10–15 first-row-atoms are about as large as can be handled accurately.

Predicting pK_a s and Other Applications

One of the molecular properties that computational chemistry is relatively good at predicting is pK_a values. There have been quite a few publications in this area. We cite only a few examples (151,152,153,154,155). (In addition, see our discussion of the SPARC approach in the next section.) Basically, the problem entails calculating the energy to dissociate a proton from the acidic functionality of a molecule. Even dissociation from nonacidic groups can be modeled. Of course, including the effect of solvation is critical. Most calculations of pK_a have been done quantum mechanically, but it is also possible to do separate molecular dynamics simulations on the parent molecule and the dissociated species. CAChe Software (93) promotes their quantum chemistry program for its ability to compute pK_a s.

Many computational chemists have done calculations to study reaction mechanisms including determining the structures of transition states and intermediates. However, there are relatively few reviews of this area according to SciFinder searches of the Chemical Abstracts Service's CPlus database (156). We can cite some examples of studying reaction energy profiles (157,158,159,160,161,162,163,164). If the reaction mechanism involves radicals, then trying to do calculations on the system becomes radically more difficult. There are relatively few papers in this area, and they generally do not pertain to pharmaceuticals (165).

Quantitative Structure–Property Relationships

Computer models can be built using two general approaches. In a data-driven approach, a large collection of data is assembled which represents the domain of the phenomenon one wishes to model. These data are then empirically fit to an equation or set of equations. Statistical,

curve-fitting and/or principal components analysis approaches are used to build an empirical model of the phenomenon. So far, we have primarily considered theoretical approaches. Let us look at data-driven approaches, and then at a way to combine the two approaches.

The data-based approach is essentially that of a quantitative structure–property [QSPR (166)] or structure–activity [QSAR (167,168)] relationship. It is a statistical correlation of measured and/or computed data with some molecular property of interest for a series of molecules. It is an arithmetic correlation, without a necessarily direct physicochemical correlation to an intrinsic property (or properties) of the molecules. The correlation is empirical. An inherently obvious limitation is that, if the model is applied to a new molecule whose structure is substantially outside the domain of the training data set used to build the model, the prediction of the model will be minimally valid, or possibly outright wrong.

Such a very early approach to modeling physical chemical properties of organic molecules was the Hammett σ – ρ linear free energy approach for predicting pK_a values (169). A more complete description of this approach, referred to more generally as the Hansch analysis approach, is given in Chapter 1 of a 1979 book (170). Forcing the measured pK_a values of a series of substituted benzoic acids to a linear model permitted proposing a series of fitted σ values for various functional group substituents and positions on the aromatic ring of benzoic acid—in effect a reverse model, determining numerical values of the σ constants that gave a linear fit. These fitted substituent values were then applied to predicting substituent effects on physical properties, and even on reactivities, of other chemical systems. Iterative refinements of these constants appeared in the literature over the years. Some major modifications were made, creating variant scales, such as the σ^+ scale, when the system to which the approach was being applied varied significantly from the original benzoic acid system. Applying the empirically determined σ or σ^+ values to predict behavior in other chemical situations worked reasonably well. However, this illustrates the flaw in empirically based models—one is at risk of attempting to naively apply the model outside its design boundaries.

Free–Wilson “additivity rule” models (168) have also been applied relatively successfully to a number of areas. Spectral interpretation—in particular, UV-visible spectral interpretation—and its correlation to molecular structure has been treated this way (171). For a given basic structure, additive factors have been empirically determined to predict effects that various functional groups have on $\lambda_{\max}$. The Bull and Breese hydrophobicity index (172) similarly calculates the relative hydrophobicity of peptides by summing contributions of the amino acid residues composing a peptide. Using multiple parameters to describe molecules, and then modeling based on the combination of properties, extends this empirical approach. A now classic successful example is the “rule of five” model for compound “druggability” (173). Lipinski’s rule of five posits that an orally active drug usually has no more than one violation of the following four criteria: (i) not more than 5 hydrogen bond donors, that is, nitrogen or oxygen atoms with one or more hydrogen atoms; (ii) not more than 10 hydrogen bond acceptors, that is, nitrogen or oxygen atoms; (iii) molecular weight under 500 daltons; and (iv) an octanol–water partition coefficient ($\log P_{o/w}$) less than 5. Predictions mostly come from regression equations or neural networks that are trained on experimental data. The amount of data and reliability of the predictions varies from excellent for pK_a , $\log P$, and solubility to more limited reliability for properties such as $\log BB$ (blood/brain partitioning) and IC_{50} for K^+ channel blockage (related to cardiac Q–T elongation) (174).

The Pennsylvania State University group reviewed early successes and failures of applications of pattern recognition approaches to building predictive models in their 1979 book (170). Much of the early work was in attempting to use measured physical properties and/or substructure classifiers to build binary-type models—to be able to classify whether a molecule belonged to a particular group or did not belong: differentiation of molecules into inhibitors versus noninhibitors, tranquilizers versus sedatives, antitumor compounds versus non-anti-tumor compounds, and other examples. These classification schemes made use of many of the statistical computational techniques that are now recognized collectively as chemometrics. The work

to that time was largely based on arbitrarily designated parameters to classify molecules. The Penn State group developed a software system, called ADAPT, which could generate a series of molecular structure descriptors. Principal component analysis was used trying to find what structure descriptors were most relevant to a model that discriminated between structures belonging to a class of compound and those that did not belong.

SPARC (SPARC Performs Automated Reasoning in Chemistry) is a computer program developed to analyze molecular properties and reactions in the same manner as an expert chemist would (175). Lionel Carreira has led efforts developing and exploiting this particular approach. SPARC does not do “first principles” computations (176). Rather, SPARC utilizes an extensive empirically derived knowledge base of organic chemistry. SPARC algorithms are based on chemical structure theory to calculate enthalpies of formation. Molecular structures are first “perceived,” and then broken into simple functional units with intrinsic properties. Units are identified as primary reaction centers or attached perturbers. In this way, it is also similar to the Hansch analysis, Hammett–Taft linear free energy, and Free–Wilson approaches. Each primary unit is analyzed and the effects of appended modifying molecular structures are quantified through perturbation theory. SPARC uses linear free energy relationships to compute thermodynamic and thermal properties, and perturbed MO methods to calculate quantum chemical effects.

Although the title of a 1991 paper by Carreira’s group implied progress in predicting chemical reactivity by computer (177), these early developments of SPARC concerned prediction of pK_a , UV-visible spectra, equilibrium constants, and some hydrolysis rate constants. Carreira’s premise is that computation of physical constants is a prerequisite to developing the means to predict kinetics. While equilibrium constants depend on the energy differences between thermodynamic states, reaction rates at which different states interconvert depend on the energy differences between the reactant (or product) and the transition state connecting the thermodynamic states. Ab initio computations are not capable of reliably computing these differences. Perturbation theory approaches, however, attempt to calculate these differences.

SPARC predicts ionization pK_a , electron affinity and numerous physical properties. The pK_a calculator was initially parameterized using measured ionization constants for 775 compounds from IPUAC data files, with a 0.22 pK_a unit root-mean-square deviation within the set. Tests on calculation of 4000 other pK_a values of known compounds (including compounds with multiple pK_a values) yielded a root-mean-square deviation of 0.35 units. This value is comparable to experimental error in measuring pK_a . Tests on specific classes of molecules show some larger variations, but still comparable to those seen in experimental measurements. Comparable rigor is shown for computation of electron affinities, and of the physical properties contributing to and leading up to similar prediction of gas chromatographic retention indices on a specific gas chromatographic liquid phase. Further validation and verification of the SPARC model (178) and predictions of chemical reactivity and physical properties (179) have been reported.

Katritzky and coworkers have explored structure-property and structure-activity relations using a multidimensional approach (1,166). Generating a number of molecular descriptors and correlating them with gas chromatographic response factors of a collection of compounds was relatively successful (180,181). They introduced the CODESSA (Comprehensive DEscriptors for Structural and Statistical Analysis) approach, where up to 1000 defined molecular descriptors can be calculated, based upon a semiempirical quantum chemical optimization of a molecular geometry, and can be correlated with experimental results to find a structure–property correlation. Using this approach, Katritzky et al. successfully modeled GC response factors, GC retention times, melting and boiling points and water-octanol partition coefficients (182). They elaborate further on the advantages of using quantum chemical calculation-generated molecular descriptors (183). The CODESSA approach has been applied to defining structure–affinity relationships for two pharmaceutically relevant receptor systems (184,185).

The CODESSA approach is a viable method to search for a computable parameter, or (more likely) a set of computable parameters, that reflect a particular type of instability in a molecular structure. Using this chemometric approach, important indicators are combined to build computational stability models. Some recent problems that have been addressed by the CODESSA approach are cited (186,187,188).

In summary, computational chemistry can be used in conjunction with standard statistical methods to find correlations between computed descriptors and molecular properties of interest. Descriptors from theoretical chemistry can be used alone or in combination with traditional parameters from physical organic and structural chemistry. If correlations exist and can be found, they can be used to predict properties of new molecules similar to but not in the training set. As with any statistical treatment, the user must be wary of overfitting experimental data with too many descriptors ("independent variables" in the language of statistics).

Expert Systems

Expert systems are computer programs that attempt to manifest the deep experience and judgment of experts in a field. Others can use the programs to evaluate problems within the domain of the rules of the expert system. Why should we now turn to an examination of expert systems? Thus far, we have been examining the basis for using computed models to make predictions about molecules. Our examination has been introductory, and experience is needed to learn about the details of the methods so that they may be used rationally, confidently, and correctly. An expert system for predicting stability would be a desirable, long-term goal. In this final section of this chapter, we review what has been accomplished toward this goal.

Chemists began exploring the use of artificial intelligence to help with synthesis planning in the 1970s. The goal was to reduce to simple computer algorithms all the rules, knowledge, and considerations that an experienced synthetic organic chemist would employ in devising the reactions necessary to reach a desired endpoint. Then other chemists could, in principle, use the programs to design synthetic routes as clever as the experts. Matthew Todd (38) reviews the development of the more successful of the synthetic predictive systems, drawing an interesting parallel between the computer-aided organic synthesis problem and efforts by various computer science groups—IBM Corporation's Deep Blue project, in particular—to develop a world-class expert system for playing chess.

Chess play is relatively simple compared to the design of a synthesis of an interesting organic molecule, or predicting how it might react. (Maybe not so simple – checkers has just recently been "solved," (189) and checkers is simpler than chess.) The problems, however, are not without some similarity. They operate on a set of "relatively simple" rules—a finite number in chess, but a not-so-finite and perhaps not-so-simple number in organic chemistry. Both problems rapidly generate a large tree of possibilities—the combinatorial explosion. Scoring is required to objectively evaluate the best route. There are likely multiple "good" paths, but how to evaluate and determine which path is the best presents problems. The analogy breaks down, however. Chess moves are binary—the result is clear-cut. Synthetic transformations and reactions have associated variable yields. A good synthetic transformation may be good mechanistically, but have a poor yield. A degradation reaction may be minor but produce a significant degradant. Rules for chess moves are unchanging. Organic chemical transformation rules are always changing, subject to conditions, and being added to with new reactions and catalysts.

Computers have become faster and more powerful. "Is it true to say that the inexorable rise in computing power will transform what are complex problems of today into trivial problems in the future? No!" (38). Deep Blue's success was not only due to speed, but also the algorithms necessary to evaluate possibilities for their relative merits. Exhaustive examination of possibilities is not sufficient. The uniquely human ability to use insight and intuition to evaluate possibilities, based on prior experiences, is what is missing. Of the 400 possible board configurations after the first round of chess play, a player of moderate experience already knows

that the majority of those configurations are not productive, and no further consideration is needed. A good organic chemist will also know by experience, which routes of transformation are productive and which are not. "Expecting computers to become creative simply by making them faster is unreasonable if we are not first creative in the way we program them" (38).

Computer-Aided Synthesis—LHASA, ORAC, IGOR, and EROS

The E. J. Corey group at Harvard started developing an expert system that was to become LHASA (Logic and Heuristics Applied to Synthetic Analysis) to help organic synthesis planning in 1967 (190,191,192,193). [Clarification is needed here. The name used for this expert system and the name of the not-for-profit research corporation, Lhasa Ltd. (mentioned later) should not be confused. They are not interchangeable, although people and concepts involved in the expert systems developed by Lhasa Ltd. are interconnected.] Collaborative and/or mentor–student relationships concerning LHASA have spawned several of the subsequent synthesis planning systems—ORAC (194) and CAMEO (195) (discussed below) specifically. LHASA was retrosynthetic in strategy, working backward from a target structure. Interactively, the scientist using the system picked a promising precursor, used it as the new target, and predicted precursors that could be transformed into it. By iteration through this cycle, a scientist can conduct reverse transformations until the precursor is a known or readily available molecule. The utility of such a system was dependent on the quality of the rules—the heuristics—captured by the system. Left to its own devices, such a system would generate a large number of "moves" directly analogous to the combinatorial explosion problem in chess. In LHASA, the combinatorial explosion problem is managed by the interactive nature of the system.

ORAC (Organic Reactions Accessed by Computer), developed by the A. Peter Johnson group in England in the 1980s, was, by contrast, a database-oriented expert system. Its primary focus was reaction retrieval from a database of transformations (39). Its success was dependent upon the quality of the associated database of information, as well as the sophistication of the facilities developed to search this database. The database was generated from the chemical literature and from cooperation by a consortium of cooperating synthetic chemists, who made available their own personal indexes of synthetic transformations.

IGOR (Interactive Generation of Organic Reactions) (196) developed by the Ugi group in Munich, Germany, used another approach in expressing molecular structure and reactions. Reactions of ensembles of molecules were treated as isomerizations. Mathematically, the ensembles were represented in a matrix notation. Mathematical manipulations were then applied in order to predict chemical transformations. While theory successfully predicts unusual chemical transformations that were subsequently verified in the laboratory, a problem with the method was that all of the atoms involved in a transformation need to be accounted for, including byproducts such as water and salts. The formalism lent itself to exhaustive investigation of all possible synthetic routes, but suffered from the combinatorial explosion problem.

EROS (Elaboration of Reactions for Organic Synthesis) (197), a program developed by Johann Gasteiger's group in Germany, addressed the IGOR combinatorial explosion by using formal rules and some energetics considerations—reaction enthalpies—to define reactive sites. Thus, only "isomerizations" at reactive sites were retained in a reaction tree for further consideration. EROS was also an interactive program, so that the combinatorial explosion was managed in part by a chemist's interaction, making selective choices to prune the tree of possibilities. The Gasteiger group has applied and extended EROS to the prediction of degradation of molecules (198,199). Components are introduced that evaluate the reactivity and potential kinetic importance of reactions, in addition to their feasibility. Estimates of kinetic expediency of reactions are based upon experimental studies of model systems.

Research activity on synthetic planning systems has subsided in the last few decades. However, as described next, efforts to develop specialized expert systems for degradation chemistry and metabolism have been pursued.

Reactivity and Degradation—CAMEO, DELPHI, and ROBIA

William L. Jorgensen's research group published a series of papers, starting in 1980, on the development of a computer expert system, CAMEO (Computer-Assisted Mechanistic Evaluation of Organic reactions) for modeling and predicting organic chemical reactivities (200). A summary of the work was published in 1990 (201). The most recent publication reporting on enhancements to CAMEO appeared in 1995 (202). Work to extend this system appears to have largely stopped.

CAMEO was originally intended as an help to planning organic syntheses. Jorgensen (201) differentiates the CAMEO efforts from the other synthesis-assisting programs. LHASA and others are based upon a retrosynthetic approach. The search is conducted over a database of known synthetic transformations, analogous to the commercial REACCS database, developed by Molecular Design Limited (203) and the database compiled by the Chemical Abstracts Service (204). The comprehensiveness of the search result is dependent on the completeness of the database. CAMEO, by contrast, was designed to approach from the complementary direction by evaluating the structure of molecules for potential reactivity toward undergoing a transformation. A molecule was evaluated based upon the computerized perception of its structure, identification of the sites of reactivity within the structure, and application of a series of mechanistic rules, derived from the literature, to predict products expected to be produced by a reaction. CAMEO had no dependence on a database of information and its level of comprehensiveness. Program functionality was dependent, rather, on addition of mechanistic rules to include reactivity situations. Base-catalyzed/nucleophilic, acid-catalyzed/electrophilic, pericyclic, oxidative/reductive, free radical and carbenoid reactions were included as of the 1990 review (201). Consideration of three-dimensional features of structures was added in 1993 (205). The last reported addition concerned the addition of Cram's Rule on stereochemical outcomes of nucleophilic addition to a carbon center with an adjacent chiral site, published in 1995 (202).

Because of CAMEO's forward synthetic rather than retrosynthetic approach, there was a heavier reliance on the creativity of the chemist to propose chemical transformations that would head in the direction of a molecule targeted for synthesis. However, once a transformation was in mind, CAMEO would provide an evaluation of the feasibility of the reaction. The concept of using CAMEO (in the mid-1990s) to predict degradative reactivity of molecules was introduced.

This seemed to be a natural application to evaluating the reactivity of molecules toward degradation—the stress-testing scenario—since CAMEO was designed based upon a forward-looking concept. Experience and pitfalls of such an application of CAMEO are discussed by Alsante and Baertschi (206). Predictions often stopped at the primary degradation product(s). Predictions might overlook secondary or ternary degradants, which may be the degradants that are actually seen in experiments. CAMEO was particularly successful at predicting certain types of chemistries—e.g., amide hydrolysis, oxidation at benzylic sites, sulfide and sulfone formation, imine formation and subsequent cleavage—but not good at others. If a molecule being analyzed had more than one reactive functional group, CAMEO would conduct its predictive analysis at the theoretically most reactive (labile?) site and ignore other sites. The original CAMEO (running on the VAX/VMS and Macintosh operating systems) was available commercially. A plug-in was available for a version of Cambridge-Soft's ChemOffice (207).

DELPHI (Degradation Expert Leading to PHarmaceutical Insight) was also described (208) as a forward-looking expert system, perceiving and assessing an input molecular structure for substructures, reactive sites and functional groups, then predicting reaction products under given conditions, based upon known general chemical principles and specialized example rules. In contrast to CAMEO, DELPHI was specifically designed to predict reactivity and degradation of molecules. DELPHI also proceeded beyond a primary reactive degradant, to subsequent degradants of degradants. Even though described in the literature, DELPHI is a proprietary software system at Pfizer.

The Goodman group proposes that their program, called ROBIA (Reaction Outcome By Informatics Analysis) (209) is the first attempt to combine heuristics-based algorithms for prediction of reactivity with molecular modeling (molecular mechanics and quantum mechanics) for evaluation of predicted products. It is a forward-looking system, basing its predictions of reactions and reactivity on properties of the starting molecule. Two examples were examined—predictions for reactions leading to the formation of dolabriferol, a natural product molecule, and intramolecular Diels–Alder formation of the steroid skeleton nucleus. They report favorable results, consistent with experimental results available on the two examples. Computations took from minutes to hours on a desktop computer with reasonable modern specifications.

Predicting Transformations—METEOR, PPS/MEPPS, and ZENETH

METEOR (210,211,212) is a knowledge-based expert system containing rules and examples relating structure and metabolic biotransformation. It is used to predict the metabolic fate of a query chemical structure under consideration. Rules contained in the intelligence base are descriptions of generic descriptions of known metabolic transformations derived from the metabolic literature, rather than simply specific entries in a database. METEOR development began in 1997, and the software is commercially available and maintained by Lhasa Ltd (213).

Judson and associates have described their implementation of the logic of argumentation (214,215) and how they have applied it in METEOR (216,217). The reasoning uses two types of rules. Absolute reasoning rules evaluate the likelihood of a biotransformation—probable, plausible, equivocal, doubted, and improbable—based upon perceived structural similarity of the query structure to generic structures in rules. Relative reasoning rules assign priorities to potentially competing reactions, equal priority being assigned when no preference is known. The reasoning engine uses further rules to avoid the potential combinatorial explosion of predictions from unconstrained analysis of a query structure. Logic of argumentation is capable of including statistical methods as a subset of the logic so that, where adequate data are available, a numerical estimate can be generated (215).

An assessment of METEOR's capabilities against a set of ten xenobiotic compounds (pharmaceuticals and industrial chemicals) has been reported (210). A total of 137 primary metabolic reactions were predicted and/or found experimentally. Correct predictions represented 30% of the cases, false positives 62%, and false negatives 8%. (Are false positives bad? With expert systems, the desire may be for the system to allow a human expert to decide whether the result really is a false positive. It is better to be alerted to the possibility and dismiss it rationally than to have never considered it at all.) The 70% discrepancies were further analyzed as to the source of the errors, and the information used to improve METEOR's capabilities and reliability.

PPS (Pathway Prediction System) is a system for predicting microbial metabolism of compounds (218,219,220). A property of METEOR is its current limitation to mammalian (eukaryotic?) metabolism. PPS addresses microbial metabolism and predicts degradation of molecular structures by microbial precedent. As such, it fills a need that METEOR does not satisfy. PPS is openly accessible and usable on the Internet (221). PPS combines a database approach with a perception of the functional groups and rule matching for a submitted structure, to justify predictions of the exact compounds in the database and of related structures. PPS correctly finds 50 (60%) of the 84 test compounds submitted for analysis, for which there are known, documented microbial biodegradation pathways. Rules to cover the inadequately predicted compounds in the test set and rules to describe new microbial biotransformations are being added.

MEPPS (MEteor Pathway Prediction System) is a combination/adaptation of the METEOR software infrastructure and PPS by replacing mammalian metabolism rules with microbial metabolism rules assembled in PPS. The adaptation is being undertaken through a collaborative effort between the University of Minnesota originators of PPS and Lhasa, Ltd.

ZENETH is the only commercially available program designed specifically for the purpose of predicting the potential degradation pathways of pharmaceutical compounds (222). It was developed by Lhasa Ltd. in consortium with a group of pharmaceutical companies. In its simplest conception, the expert system substitutes chemical transformation rules for the metabolic transformation rules of METEOR to create a chemical degradation predictor, much like CAMEO and DELPHI did. The success of ZENETH will be revealed as users gain experience with it.

Toxicity Predictions—DEREK, MCASE and TOPKAT

Three expert systems exist addressing the issues surrounding genotoxicity of new chemical entities. Rather than predicting reactivity, these systems perceive and evaluate query chemical structures for the presence of substructural motifs that are known to or expected to cause toxicity.

DEREK (Deductive Estimation of Risk from Existing Knowledge) makes qualitative estimations of genotoxicity potential of organic structures. Development started in 1989 (215). The system identifies whether a known DNA-reactive structural moiety is present in a test molecule. Evaluations are made with reference to a database of published bacterial mutagenicity and other available genotoxicity data. An extensive evaluation of the validity and limitations of DEREK predictions has been published (223). DEREK is unique among the three genotoxicity evaluation systems in that its intelligence is broadly based to include all available genotoxicity data, not just bacterial mutagenicity. Even though DEREK identifies and alerts of a possible toxicity issue, final judgment is still dependent upon and left to an expert user to determine if the alert found is in the proper chemical context, relative to the compounds on which the DEREK rule was based. A human expert interpreting the indications found by the expert system is a critical and essential component. In 1999, a predictive assessment of DEREK's accuracy was published (215). Of the 266 chemicals considered valid for testing, 81 of 114 mutagens (71%) and 117 of 152 nonmutagens (77%) were correctly identified. The results of this analysis prompted development of additional rules which increased correct predictions to 96 (84%) of the 114 mutagens. The *Salmonella* mutagenicity assay itself is acknowledged to only be 85% accurate.

MCASE (Multiple Computer Aided Structure Evaluation), another artificial intelligence evaluation tool, uses a different approach to evaluating a submitted molecular structure (224). It "dissociates" a test molecule into 2- to 10-atom fragments (termed "biophores" by the software developers) and statistically evaluates the strength of association of the fragments with mutagenicity, generating a quantitative prediction that is refined through taking into consideration physicochemical properties and the existence of potentially deactivating fragments. For certain versions of MCASE, the learning set is based solely on bacterial mutagenicity data derived from 2032 compounds, most of which are environmental toxicants, only 204 of which are pharmaceuticals.

TOPKAT (225,226) (TOxicity Prediction by Komputer-Assisted Technology), which was developed by Kurt Enslein's company, Health Designs, uses electrotopological descriptors rather than chemical structures to predict mutagenic reactivity with DNA and is an extension of classical quantitative structure-activity relationship analysis. TOPKAT intelligence is derived solely from bacterial mutagenicity data and produces a probability that a submitted compound could present genotoxicity issues.

Other evaluations of these systems' performance have been reported in addition to the original authors'. Snyder et al. (223) present results of their evaluation which are not nearly as favorable as the 1999 evaluation of DEREK. Their evaluations indicate a generally poor sensitivity of all three expert systems for predicting Ames test positivity (43% to 52% sensitivity) and poorer sensitivity in predicting other genotoxicities, suggesting that these systems do not provide sufficient predictivity to be of value in routine drug safety applications. A second study reports the evaluation of eight structure-activity-based predictive models for toxicity (227) including DEREK and TOPKAT. Overall, the exercises showed that in all cases, and consistent

with the previous study (223), the best performance was attained by approaches that relied largely (and ultimately) on human expert judgment. In direct comparisons, human expert judgment has always outperformed the automatic prediction systems. Unfortunately, human expert judgment requires a large amount of individual, subjective skill. One conclusion is that the currently available software packages do not sufficiently integrate and exploit information and data, which can be better handled by human experts (227).

CONCLUSIONS

Computational chemistry offers many tools for examining research questions arising in stress testing of pharmaceuticals. The methods and software have become easy to use and are progressing in ability, some more than others. Whereas they cannot answer many questions faced by the experimentalist, they can give the researcher additional advantage in many research and development situations. The tools can be used for studying a wide range of molecules with regard to reaction pathways, conformations, electronic structure, and other physicochemical properties.

Pharmaceutical stress-testing beckons with many novel and unique opportunities for applying computational chemistry. Our effort on writing this chapter will be well rewarded if some of our readers are encouraged to try some of the many techniques of computational chemistry. Computers and software for computational chemistry are available at many companies and universities. Additionally, the tools are inexpensive enough that many individuals may acquire them. Our basic inquisitiveness as scientists stimulates us to use those tools that elucidate difficult or otherwise insoluble research questions.

As with any scientific tool, computational chemistry results must be used and interpreted with some understanding. A primary purpose of this chapter has been to give a wide-ranging practical introduction and assessment. Gaining experience with the tools will allow the user to phrase research questions in the most effective way.

The beginner need not be intimidated. There may be computational chemists already employed at your company or university. Contact them and see if they have an interest in collaborating on some research problems you have in mind. Also, check out computational chemists at local colleges and universities. They may be willing to share their time and expertise helping you. Even if these other scientists may not be in a position to collaborate, they may have time to give advice or answer specific questions about the software, methodology, design of computational experiments, and/or meaning of the computer output. In addition, anyone can subscribe free to the Computational Chemistry List (228), a listserv. Subscribers can post questions. A pool of several thousand computational chemistry experts and users are available to share their knowledge. Whereas the experts will not be willing to read a software manual for you, they will give help to those who make a genuine effort to get started and perform some calculations.

One sure prediction is that the use of modern computers and software will continue to increase as more scientists discover the value of computational chemistry for particular research problems.

ACKNOWLEDGMENTS

We are grateful to Dr. Karen Alsante and Dr. Steven Baertschi for their editorial suggestions and patience.

REFERENCES

1. Katritzky AR, Karelson M, Lobanov VS. SPR as a means of predicting and understanding chemical and physical properties in terms of structure. *Pure Appl Chem* 1997; 69: 245–8.
2. Boyd DB. The power of computational chemistry to leverage stress testing of pharmaceuticals. Chapter 12. In: Baertschi SW, ed. *Pharmaceutical Stress Testing: Predicting Drug Degradation*. Boca Raton, FL: Taylor and Francis, 2005: 355–418.

3. Young DC. Computational Chemistry: A Practical Guide for Applying Techniques to Real-World Problems. New York: Wiley-Interscience, 2001.
4. Boyd DB. Molecular Modeling Software in Use: Publication Trends. In: Lipkowitz KB, Boyd DB, eds. Reviews in Computational Chemistry. Vol. 6. New York: VCH, 1995: 317–54.
5. Lipkowitz KB, Boyd DB, eds. Reviews in Computational Chemistry. Vol. 8. New York: VCH, 1996: v–ix.
6. Lipkowitz KB, Boyd DB, eds. Reviews in Computational Chemistry. Vol. 17. New York: VCH, 2001: v–xxviii.
7. Bachrach SM. Computational Organic Chemistry. Hoboken, NJ: Wiley, 2007.
8. Lipkowitz KB, Boyd DB, eds. Reviews in Computational Chemistry. Vol. 1. New York: VCH, 1990: vii–xii.
9. Hopfinger AJ. Computer-assisted drug design. *J Med Chem* 1985; 28: 1133–9.
10. Leach AR. Molecular Modeling: Principles and Applications, 2nd edn. Harlow, UK: Pearson Education, 2001.
11. Van de Waterbeemd H, Carter RE, Grassly G, et al. Glossary of terms used in computational drug design. *Pure Appl Chem* 1997; 69: 1137–52.
12. Jensen F. Introduction to Computational Chemistry. Chichester, UK: Wiley, 1999.
13. Boyd DB, Lipkowitz KB. Examination of the employment environment for computational chemistry. In: Lipkowitz KB, Boyd DB, eds. Reviews in Computational Chemistry. Vol. 18. 2002: 293–319.
14. Boyd DB. Evidence that there is a future for semi-empirical calculations. *J Mol Struct: THEOCHEM* 1997; 401: 219–25.
15. Lindley D. Uncertainty: Einstein, Heisenberg, Bohr and the Struggle for the Soul of Science. New York: Anchor Books, 2007.
16. Szabo A, Ostlund NS. Modern Quantum Chemistry: Introduction to Advanced Electronic Structure Theory. Mineola, New York: Dover Publications, 1989.
17. Cook, DB. Handbook of Computational Quantum Chemistry. Mineola, NY: Dover Publications, 1998.
18. McMahon D. Quantum Mechanics Demystified. New York: McGraw-Hill, 2006.
19. Lipkowitz KB, Boyd DB. Appendix: Books published on the topics of computational chemistry. In: Lipkowitz KB, Boyd DB, eds. Reviews in Computational Chemistry. Vol. 17. New York: Wiley-VCH, 2001: 255–357.
20. Hehre WJ, Radom L, Schleyer PvR, Pople JA. Ab Initio Molecular Orbital Theory. New York: Wiley-Interscience, 1986.
21. Pople JA, Beveridge DL. Approximate Molecular Orbital Theory. New York: McGraw-Hill, 1970.
22. Cramer, CJ. Essentials of Computational Chemistry: Theories and Models. Chichester, UK: Wiley, 2002.
23. Lewars E. Computational Chemistry: Introduction to the Theory and Applications of Molecular and Quantum Mechanics. Boston: Kluwer Academic, 2003.
24. Hinchliffe A. Molecular Modeling for Beginners, 2nd ed. Chichester, UK: Wiley, 2007.
25. Klose RT. The joys of science. *Newsweek*, 26 October 1987.
26. Isaacson W. Einstein: His Life and Universe. New York: Simon & Schuster, 2007.
27. Jones S. The Quantum Ten: A Story of Passion, Tragedy Ambition and Science. New York: Oxford University Press, 2008.
28. Boyd DB, Lipkowitz KB. History of the Gordon Research Conferences on Computational Chemistry. In: Lipkowitz KB, Boyd DB, eds. Reviews in Computational Chemistry, Vol. 14. New York: Wiley-VCH, 2000, 399–439.
29. Hendrickson JB. Molecular geometry. I. Machine computation of the common rings. *J Am Chem Soc* 1961; 83: 4537–47.
30. Levitt M, Lifson S. Refinement of protein conformations using a macromolecular energy minimization procedure. *J Mol Biol* 1969; 46: 269–79.
31. Allinger NL. Conformational analysis. 130. MM2. A hydrocarbon force field utilizing V1 and V2 torsional terms. *J Am Chem Soc* 1977; 99: 8127–34.
32. Bolcer JD, Hermann RB. The development of computational chemistry in the United States. In: Lipkowitz KB, Boyd DB, eds. Reviews in Computational Chemistry. Vol. 5. New York: VCH, 1994: 1–63.
33. Smith SJ, Sutcliffe BT. The development of computational chemistry in the United Kingdom. In: Lipkowitz KB, Boyd DB, eds. Reviews in Computational Chemistry. Vol. 10, New York: VCH, 1997: 271–316.

34. Rivail JL, Maigret B. Computational chemistry in France: A historical survey. In: Lipkowitz KB, Boyd DB, eds. *Reviews in Computational Chemistry*. Vol. 12. New York: VCH, 1998: 367–80.
35. Boyd RJ. The development of computational chemistry in Canada. In: Lipkowitz KB, Boyd DB, eds. *Reviews in Computational Chemistry*. Vol. 15. New York: Wiley-VCH, 2000: 213–99.
36. Peyerimhoff SD. The development of computational chemistry in Germany. In: Lipkowitz KB, Boyd DB, eds. *Reviews in Computational Chemistry*. Vol. 18. Hoboken, NJ: Wiley-VCH, 2002: 293–319.
37. Hubbard RE. Molecular graphics and modeling: Tools of the trade. In: Cohen NC, ed. *Guidebook to Molecular Modeling and Drug Design*. San Diego, CA: Academic Press, 1996: 19–54.
38. Todd, MH. Computer-aided organic synthesis. *Chem Soc Rev* 2005; 34: 247–66.
39. Johnson AP. Computer aids to synthesis planning. *Chem Brit* 1985; 21: 59–67.
40. <http://www.symyx.com>
41. <http://www.daylight.com>
42. Allen FH, Davies JE, Galloy JJ, et al. The development of versions 3 and 4 of the Cambridge structural database system. *J Chem Inf Comput Sci* 1991; 31: 187–204.
43. Müller K. Molecular modeling and structural data bases in pharmaceutical research. In: Jensen B, Jørgensen FS, Kofod H, eds. *Frontiers in Drug Research-Crystallographic and Computational Methods*, Alfred Benzon Symp. No. 28. Copenhagen: Munksgaard, 1989: 210–21.
44. Boyd DB. Aspects of molecular modeling. In: Lipkowitz KB, Boyd DB, eds. *Reviews in Computational Chemistry*. New York: VCH, 1990: 321–54.
45. <http://www.ccdc.cam.ac.uk>.
46. <http://pubchem.ncbi.nlm.nih.gov/>
47. Boyd DB, Lipkowitz KB. Molecular mechanics. The method and its underlying philosophy. *J Chem Educ* 1982; 59: 269–74.
48. Dinur U, Hagler AT. New Approaches to Empirical Force Fields. In: Lipkowitz KB, Boyd DB, eds. *Reviews in Computational Chemistry*. Vol. 2. 1991; 2: 99–164.
49. DeKock RL, Madura JD, Rioux F, Casanova J. Computational chemistry in the undergraduate curriculum. In: Lipkowitz KB, Boyd DB, eds. *Reviews in Computational Chemistry*, Vol. 4. New York: VCH, 1993, 149–228.
50. Landis CR, Root DM, Cleveland T. Molecular mechanics force fields for modeling inorganic and organometallic compounds. In: Lipkowitz KB, Boyd DB, eds. *Reviews in Computational Chemistry*. Vol. 6. New York: VCH, 1995: 73–148.
51. Burkert U, Allinger NL. *Molecular Mechanics*. ACS Monograph 177. Washington, DC: American Chemical Society, 1982: 339.
52. Allinger NL. Molecular mechanics. In: Domenicano A, Hargittai I, eds. *Accurate Molecular Structures: Their Determination and Importance*. Monographs on Crystallography. Vol. 1. Chester, UK: International Union of Crystallography and Oxford: Oxford University Press, 1992: 336–54.
53. Allinger NL, Yuh YH, Lii JH. Molecular mechanics. The MM3 force field for hydrocarbons: 1. *J Am Chem Soc* 1989; 111: 8551–66.
54. Lii JH, Allinger NL. Molecular mechanics. The MM3 force field for hydrocarbons. 2. Vibrational frequencies and thermodynamics. *J Am Chem Soc* 1989; 111: 8566–75.
55. Lii JH, Allinger NL. Molecular mechanics. The MM3 force field for hydrocarbons: 3. The van der Waals' potentials and crystal data for aliphatic and aromatic hydrocarbons. *J Am Chem Soc* 1989; 111: 8576–82.
56. Allinger NL, Chen K, Lii JH. An improved force field (MM4) for saturated hydrocarbons. *J Comput Chem* 1996; 17: 642–68.
57. Nevins N, Chen K, Allinger NL. Molecular mechanics (MM4) calculations on alkenes. *J Comput Chem* 1996; 17: 669–94.
58. Nevins N, Lii JH, Allinger NL. Molecular mechanics (MM4) calculations on conjugated hydrocarbons. *J Comput Chem* 1996; 17: 695–729.
59. Nevins N, Allinger NL. Molecular mechanics (MM4) vibrational frequency calculations for alkenes and conjugated hydrocarbons. *J Comput Chem* 1996; 17: 730–46.
60. Allinger NL, Chen K, Katzenellenbogen JA, et al. Hyperconjugative effects on carbon-carbon bond lengths in molecular mechanics (MM4). *J Comput Chem* 1996; 17: 747–55.
61. Halgren TA. The representation of van der Waals (vdW) interactions in molecular mechanics force fields: Potential form, combination rules, and vdW parameters. *J Am Chem Soc* 1992; 114: 7827–43.
62. Halgren TA. Merck molecular force field. I. Basis, form, scope, parameterization, and performance of MMFF94. *J Comput Chem* 1996, 17, 490–519.

63. Halgren TA. Merck molecular force field. II. MMFF94 van der Waals and electrostatic parameters for intermolecular interactions. *J Comput Chem* 1996; 17: 520–52.
64. Halgren TA. Merck molecular force field. III. Molecular geometries and vibrational frequencies for MMFF94. *J Comput Chem* 1996; 17: 553–86.
65. Halgren TA, Nachbar RB. Merck molecular force field. IV. Conformational energies and geometries for MMFF94. *J Comput Chem* 1996; 17: 587–15.
66. Halgren TA. Merck molecular force field. V. Extension of MMFF94 using experimental data, additional computational data, and empirical rules. *J Comput Chem* 1996; 17: 616–41.
67. Halgren TA. MMFF. VI. MMFF94s option for energy minimization studies. *J Comput Chem* 1999; 20: 720–29.
68. Halgren TA. MMFF. VII. Characterization of MMFF94, MMFF94s, and other widely available force fields for conformational energies and for intermolecular-interaction energies and geometries. *J Comput Chem* 1999; 20: 730–48.
69. Pettersson I, Liljefors T. Molecular mechanics calculated conformational energies of organic molecules: a comparison of force fields. In: Lipkowitz KB, Boyd DB, eds. *Reviews in Computational Chemistry*. Vol. 9. New York: VCH, 1996: 167–89.
70. Rogers DW. *Computational Chemistry using the PC*, 3rd edn. Hoboken, NJ: Wiley-Interscience, 2003: 349.
71. Van Gunsteren WF, Berendsen HJC. Computer simulation of molecular dynamics: Methodology, applications and perspectives in chemistry. *Angew Chem Int Ed Engl* 1990; 29: 992–1023.
72. Gubbins KE, Quirke N. *Molecular Simulation and Industrial Applications: Methods, Example and Prospects*. Amsterdam: Gordon & Breach, 1996.
73. Boyd DB. Computer-aided molecular design. In: Kent A, Williams JG, eds. *Encyclopedia of Computer Science and Technology*. Vol. 33 (Suppl 18). New York: Marcel Dekker, 1995: 41–71.
74. Lybrand TP. Computer simulation of biomolecular systems using molecular dynamics and free energy perturbation methods. In: Lipkowitz KB, Boyd DB, eds. *Reviews in Computational Chemistry*. New York: VCH, 1990: 295–320.
75. Meirovitch H. Calculation of the free energy and the entropy of macromolecular systems by computer simulation. In: Lipkowitz KB, Boyd DB, eds. *Reviews in Computational Chemistry*. Vol. 12. New York: Wiley-VCH, 1998: 1–74.
76. Balbes LM, Mascarella SW, Boyd DB. A perspective of modern methods in computer-aided drug design. In: Lipkowitz KB, Boyd DB, eds. *Reviews in Computational Chemistry*. Vol. 5. New York: VCH, 1994: 337–79.
77. Reddy MR, Erion MD, Agarwal A. Free energy calculations: Use and limitations in predicting ligand-binding affinities. In: Lipkowitz KB, Boyd DB, eds. *Reviews in Computational Chemistry*. Vol. 16. New York: Wiley-VCH, 2000: 217–304.
78. Woods RJ. The application of molecular modeling techniques to the determination of oligosaccharide solution conformations. In: Lipkowitz KB, Boyd DB, eds. *Reviews in Computational Chemistry*. Vol. 9. New York: VCH, 1996: 129–65.
79. Pross A. *Theoretical and Physical Principles of Organic Reactivity*. New York: Wiley-Interscience, 1995.
80. Lipkowitz KB, Peterson MA. Molecular mechanics in organic synthesis. *Chem Rev* 1993; 93: 2463–86.
81. Zerner MC. Semiempirical molecular orbital methods. In: Lipkowitz KB, Boyd DB, eds. *Reviews in Computational Chemistry*. Vol. 2. New York: VCH, 1991: 313–65.
82. Dewar MJS, Thiel W. Ground states of molecules. 38. The MNDO method. Approximations and parameters. *J Am Chem Soc* 1977; 99: 4899–4907.
83. Dewar MJS, Thiel W. Ground States of Molecules. 39. MNDO results for molecules containing hydrogen, carbon, nitrogen and oxygen. *J Am Chem Soc* 1977; 99: 4907–17.
84. <http://www.semichem.com>.
85. Dewar MJS, Zoebisch EG, Healey EF, Stewart JJP. AM1: A new general purpose quantum mechanical molecular model. *J Am Chem Soc* 1985; 107: 3902–09.
86. Dewar MJS, Zoebisch EG. Extension of AM1 to the halogens. *J Mol Struct: THEOCHEM* 1988; 180: 1–21.
87. Dewar MJS, Jie C. AM1 parameters for phosphorus. *J Mol Struct: THEOCHEM* 1989; 187: 1–13.
88. Dewar MJS, Yuan YC. AM1 parameters for sulfur. *Inorg Chem* 1990; 29: 3881–90.
89. Stewart JJP. Optimization of parameters for semiempirical methods: I. Method. *J Comput Chem* 1989; 10: 209–20.

90. Dewar MJS, Jie C, Yu J. SAM1: the first of a new series of general-purpose quantum mechanical molecular models. *Tetrahedron* 1993; 49: 5003–38.
91. Holder AJ, Dennington RD II, Jie C. Addendum to SAM1 results previously published. *Tetrahedron* 1994; 50: 627–38.
92. Thiel W, Voityuk AA. Extension of MNDO to d orbitals: Parameters and results for the second-row elements and for the zinc group. *J Phys Chem* 1996; 100: 616–26.
93. <http://www.cache.fujitsu.com/cache/index.shtml>
94. Rocha GB, Freire RO, Simas AM, Stewart JJP. RM1: a reparameterization of AM1 for H, C, N, O, P, S, F, Cl, Br, and I. *J Comput Chem* 2006; 27: 1101–11.
95. Stewart JJP. Optimization of parameters for semiempirical methods: V. Modification of NDDO approximations and application to 70 elements. *J Mol Model* 2007; 13: 1173–213.
96. Repasky MP, Chandrasekhar J, Jorgensen WL. PDDG/PM3 and PDDGMNDO: Improved semiempirical methods. *J Comput Chem* 2002; 23: 1601–22.
97. Tubert-Brohman I, Guimarães CRW, Repasky MP, Jorgensen WL. Extension of the PDDG/PM3 and PDDG/MNDO semiempirical molecular orbital methods to the halogens. *J Comput Chem* 2003; 25: 138–50.
98. Tubert-Brohman I, Guimarães CRW, Jorgensen WL. Extension of the PDDG/PM3 semiempirical molecular orbital method to sulfur, silicon and phosphorus. *J Chem Theor Comput* 2005; 1: 817–23.
99. Bartolotti LJ, Flurichick, K. An introduction to density functional theory. In: Lipkowitz KB, Boyd DB, eds. *Reviews in Computational Chemistry*. Vol. 7. New York: Wiley-VCH, 1995: 187–216.
100. St. Amant A. Density functional methods in biomolecular modeling. In: Lipkowitz KB, Boyd DB, eds. *Reviews in Computational Chemistry*. Vol. 7. New York: Wiley-VCH, 1995: 217–59.
101. Bickelhaupt FM, Baerends EJ. Kohn-Sham density functional theory: Predicting and understanding chemistry. In: Lipkowitz KB, Boyd DB, eds. *Reviews in Computational Chemistry*. Vol. 15. New York: Wiley-VCH, 2000: 1–86.
102. Koch W, Holthausen MC. *A Chemist's Guide to Density Functional Theory*. Weinheim, Germany: Wiley-VCH, 2001: 300.
103. http://nobelprize.org/nobel_prizes/chemistry/laureates/1998/
104. Curtiss LA, Raghavachari K, Pople JA. Gaussian-2 theory using reduced Moller-Plesset orders. *J Chem Phys* 1993; 98: 1293–8.
105. Boyd DB. Compendium of software and internet tools for computational chemistry. In: Lipkowitz KB, Boyd DB, eds. *Reviews in Computational Chemistry*. Vol. 11. New York: Wiley-VCH, 1997: 373–99.
106. <http://www.cambridgesoft.com/software/ChemDraw/>
107. Helson HE. Structure diagram generation. In: *Reviews in Computational Chemistry*. Vol. 13. Lipkowitz KB, Boyd DB, eds. New York: Wiley-VCH, 1999; 223: 313–98.
108. <http://www.symyx.com/downloads/downloadable/>
109. <http://www.acdlabs.com/download/>
110. <http://dragon.unideb.hu/~gundat/rajzprogramok/dprog.html>
111. <http://jmol.sourceforge.net/>
112. <http://www.csc.fi/english/pages/gOpenMol>
113. <http://molekel.cscs.ch/wiki/pmwiki.php/Main/HomePage>
114. Te Velde G, Bickelhaupt FM, Baerends EJ, et al. Chemistry with ADF. *J Comput Chem* 2001; 22: 931–67.
115. <http://www.chemcomp.com/>
116. Stewart JJP. MOPAC2009, Stewart Computational Chemistry, Colorado Springs, CO, USA. <http://openmopac.net/>
117. Schmidt MW, Baldridge KK, Boatz JA, et al. General atomic and molecular electronic-structure System. *J Comput Chem* 1993, 14, 1347–63.
118. <http://www.msg.ameslab.gov/GAMESS/>
119. Valiev M, Bylaska EJ, Govind N, et al. NWChem: a comprehensive and scalable open-source solution for large scale molecular simulations. *Comput Phys Commun* 2010; 181: 1477–89.
120. <http://www.nwchem-sw.org/index.php/Download>
121. Neese F. ORCA—an ab initio, density functional and semi-empirical program package, Version 2.6, 2008. University of Bonn, Wegelerstraße 12, D-53115 Bonn, Germany, <http://www.thch.uni-bonn.de/tc/orca/>
122. Bally T, Borden WT. Calculations on open-shell molecules: A beginner's guide. In: Lipkowitz KB, Boyd DB, eds. *Reviews in Computational Chemistry*. Vol. 13, New York: Wiley-VCH, 1999: 1–97.
123. Eliel EL, Wilen SH. *Stereochemistry of Organic Compounds*. New York: Wiley, 1994: 599–602.

124. Christie GH, Kenner J. The molecular configurations of polynuclear aromatic compounds. Part I: The resolution of 6,6'-dinitro- and 4,6,4,6-tetranitro-diphenic acids into optically active components. *J Chem Soc* 1932; 121: 614.
125. Eliel, EL. Wilen, SH. Stereochemistry of Organic Compounds. New York: Wiley, 1994: 1142–50.
126. Klamt A, Schüürmann G. COSMO: A new approach to dielectric screening in solvents with explicit expressions for the screening energy and its gradient. *J Chem Soc* 1993; 2: 799–805.
127. Klamt A. Conductor-like screening model for real solvents: A new approach to the quantitative calculation of solvation phenomena. *J Phys Chem* 1995; 99: 2224–35.
128. Cramer CJ, Truhlar DG. Continuum solvation models: Classical and quantum mechanical implementations. In: Lipkowitz KB, Boyd DB, eds. *Reviews in Computational Chemistry*. Vol. 6. New York: VCH, 1995: 1–72.
129. Chambers CC, Hawkins GD, Cramer CJ, Truhlar DG. Model for aqueous solvation based on class IV atomic charges and first solvation shell effects. *J Phys Chem* 1996; 100: 16385–98.
130. Marenich AV, Olsen RM, Kelly CP, Cramer CJ, Truhlar DG. Self-consistent reaction field model for aqueous and nonaqueous solutions based on accurate polarized partial charges. *J Chem Theor Comput* 2007; 3: 11–2033.
131. Boyd DB. β -Lactam antibacterial agents: computational chemistry investigations. In: Greenberg A, Breneman CM, Liebman JF, eds. *The Amide Linkage: Structural Significance in Chemistry, Biochemistry, and Materials Science*. New York: Wiley, 2000: 337–75.
132. Boyd DB. Theoretical and physicochemical studies on β -lactam antibiotics. In: Morin RB, Gorman M, eds. *β -Lactam Antibiotics: Chemistry and Biology*. Vol. 1. New York: Academic Press, 1982: 437–545.
133. Boyd DB, Hermann RB, Presti DE, Marsh MM. Electronic structures of cephalosporins and penicillins: 4. Modeling acylation by the β -lactam ring. *J Med Chem* 1975; 18: 408–17.
134. Boyd DB, Herron DK, Lunn WHW, Spitzer WA. Parabolic relationships between antibacterial activity of cephalosporins and β -lactam reactivity predicted from molecular orbital calculations. *J Am Chem Soc* 1980; 102: 1812–14.
135. Boyd DB. Substituent effects in cephalosporins as assessed by molecular orbital calculations, nuclear magnetic resonance, and kinetics. *J Med Chem* 1983; 26: 1010–13.
136. Boyd DB. Electronic structure of cephalosporins and penicillins: 15. Inductive effect of the 3-position side chain in cephalosporins. *J Med Chem* 1984; 27: 63–6.
137. Bally T, Borden WT. Calculations on open-shell molecules: A beginner's guide. In: Lipkowitz KB, Boyd DB, eds. *Reviews in Computational Chemistry*. Vol. 13. New York: Wiley-VCH, 1999: 1–97.
138. Blanksby SJ, Ellison GB. Bond dissociation energies of organic molecules. *Acc Chem Res* 2003; 36: 255–63.
139. Stewart JJP. Optimization of parameters for semiempirical methods: V. Modification of NDDO approximations and application to 70 elements. *J Mol Model* 2007; 13: 1173–213.
140. March J. *Advanced Organic Chemistry*, 4th edn. New York: Wiley-Interscience, 1992: 23–5.
141. Lewin JL, Cramer CJ. Rapid quantum mechanical models for the computational estimation of C–H bond dissociation energies as a measure of metabolic stability. *Mol Pharmaceutics* 2004; 1: 128–35.
142. Boyd RJ, Glover JNM, Pincock JA. A theoretical study of the change in hemolytic bond dissociation energy on conversion of A–B to A–B+H. *J Am Chem Soc* 1989; 111: 5152–5.
143. Brinck T, Haeblerlein M, Jonsson M. A computational analysis of substituent effects on the O–H bond dissociation energy in phenols: Polar versus radical effects. *J Am Chem Soc* 1997; 119: 4239–44.
144. Zhu X-Q, Wang C-H, Liang H. Scales of oxidation potentials, pK_a , and BDE of various hydroquinones and catechols in DMSO. *J Org Chem* 2010; 75: 7240–57.
145. Bartlett RJ, Stanton JF. Applications of post-Hartree-Fock methods: A tutorial. In: Lipkowitz KB, Boyd DB, eds. *Reviews in Computational Chemistry*. Vol. 5. New York: VCH, 1994: 65–169.
146. Fung H-L, Conway WD. A student experiment in pharmaceuticals: pH partitioning of a weak acid. *Am J Pharmaceut Educ* 1974; 38: 523–30.
147. Cheeseman JR, Trucks GW, Keith TA, Frisch MJ. A comparison of models for calculating nuclear magnetic resonance shielding tensors. *J Chem Phys* 1996; 104: 5497–509.
148. Wiberg KB, Hammer JD, Zilm KW, et al. NMR Chemical Shifts. 3. A comparison of acetylene, allene and the higher cumulenes. *J Org Chem* 1999; 64: 6394–400.
149. Minick DJ, Rutkowski RD, Miller LA. Strategies for successfully applying vibrational circular dichroism in a pharmaceutical research environment. *Am Pharmaceut Rev* 2007; 10, 118–23.
150. Robb MA, Garavelli M, Olivucci M, Bernardi F. A computational strategy for organic photochemistry. In: Lipkowitz KB, Boyd DB, eds. *Reviews in Computational Chemistry*. Vol. 15. New York: Wiley-VCH, 2000: 87–146.

151. Alongi KS, Shields GC. Theoretical calculations of acid dissociation constants: A review article. In: Wheeler RA, Spellmeyer DC, eds. *Annual Reports in Computational Chemistry*. Amsterdam: Elsevier, 2010. Vol. 6, 113–38.
152. Eckert F, Diedenhofen M, Klamt A. Towards a first principles prediction of pK_a : COSMO-RS and the cluster-continuum approach. *Mol Phys* 2010; 108: 229–41.
153. Ding F, Smith JM, Wang H. First-principles calculation of pK_a values for organic acids in nonaqueous solution. *J Org Chem* 2009; 74: 2679–91.
154. Wallace JA, Shen JK. Predicting pK_a values with continuous constant pH molecular dynamics. *Methods in Enzymology* 2009; 466 (Biothermodynamics, Part B): 455–75.
155. Yates BF. Computational organic chemistry. *Annual Reports on the Progress of Chemistry, Section B: Organic Chemistry* 2003; 99: 292–325.
156. Truhlar DG, Garrett BC, Klippenstein SJ. Current status of transition-state theory. *J Phys Chem* 1996; 100: 12771–800.
157. Chen X, Regan CK, Craig SL, Krenske EH, Houk KN, Jorgensen WL, Brauman JI. Steric and solvation effects in ionic S_N2 reactions. *J Am Chem Soc* 2009; 131: 16162–70.
158. Lim D, Jenson C, Repasky MP, Jorgensen WL. Solvent as catalyst: Computational studies of organic reactions in solution. In: Truhlar DG, Morokuma K, eds. *Transition State Modeling for Catalysis*. Washington, DC: American Chemical Society, 1999; 721: 74–85.
159. Evanseck JD, Blake JF, Jorgensen WL. Ab initio study of the S_N2 reactions of hydroxide and hydroperoxide with chloromethane. *J Am Chem Soc* 1987; 109: 2349–53.
160. Acevedo O, Jorgensen WL. Solvent effects on organic reactions from QM/MM simulations. In: Spellmeyer DC, ed. *Annual Reports in Computational Chemistry*. Amsterdam: Elsevier, 2006; 2: 263–78.
161. Jorgensen WL, Buckner JK. Effect of hydration on the structure of an S_N2 transition state. *J Phys Chem* 1986; 90: 4651–54.
162. Chandrasekhar J, Jorgensen WL. Energy profile for a nonconcerted S_N2 reaction in solution. *J Am Chem Soc* 1985; 107: 2974–5.
163. Chandrasekhar J, Smith SF, Jorgensen WL. Theoretical examination of the S_N2 reaction involving chloride ion and methyl chloride in the gas phase and aqueous solution. *J Am Chem Soc* 1985; 107: 154–63.
164. Chandrasekhar J, Smith SF, Jorgensen WL. S_N2 reaction profiles in the gas phase and aqueous solution. *J Am Chem Soc* 1984; 106: 3049–50.
165. Dibble TS. Failures and limitations of quantum chemistry for two key problems in the atmospheric chemistry of peroxy radicals. *Atmospheric Environment* 2008; 42: 5837–48.
166. Katritzky AR, Oliferenko AA, Oliferenko PV, et al. A general treatment of solubility: 1. The QSPR correlation of solvation free energies of single solutes in series of solvents. *J Chem Inf Comput Sci* 2003; 43: 1794–805.
167. Martin YC. *Quantitative Drug Design: A Critical Introduction*. New York: Marcel Dekker, 1978.
168. Plummer EL. The application of quantitative design strategies in pesticide design. In: Lipkowitz KB, Boyd DB, eds. *Reviews in Computational Chemistry*. Vol. 1, New York: VCH Publishers, 1990: 119–68.
169. Jaffe HH. A re-examination of the Hammett equation. *Chem Rev* 1953; 53: 191–261.
170. Stuper AJ, Brügger WE, Jurs PC. *Computer-Assisted Studies of Chemical Structure and Biological Function*. New York: Wiley-Interscience, 1979.
171. Silverstein MM, Bassler GC, Morrill TC. *Ultraviolet spectrometry*. Chapter 6. *Spectrometric Identification of Organic Compounds*, 4th edn. New York: Wiley, 1981.
172. Bull HB, Breese K. Surface tension of amino acid solutions: A hydrophobicity scale of the amino acid residues. *Arch Biochem Biophys* 1974; 161: 665–70.
173. Lipinski CA, Lombardo F, Dominy BW, et al. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv Drug Deliv Rev* 1997; 23: 3–25.
174. Jorgensen WL. The many roles of computation in drug discovery. *Science* 2004; 303: 1813–18.
175. Whiteside TS, Carreira LA. Prediction of the enthalpy of formation of hydrocarbons using SPARC. *J Theor Comput Chem* 2004; 3: 451–69.
176. Hilal SH, Carreira LA, Karickhoff SW. Estimation of chemical reactivity parameters and physical properties of organic molecules using SPARC. In: Politzer P, Murray JS, eds., *Quantitative Treatments of Solute/Solvent Interactions, Theoretical and Computational Chemistry*. Vol. 1. Amsterdam: Elsevier, 1994: 291–353.

177. Karickhoff SW, McDaniel VK, Melton C, et al. Predicting chemical reactivity by computer. *Environ Toxicol Chem* 1991; 10: 1405–16.
178. Hilal SH, Karickhoff SW, Carreira LA. Verification and validation of the SPARC model. U.S. Environmental Protection Agency Report EPA/600/R-03/033. Washington, DC: U.S. Environmental Protection Agency, 2003.
179. Hilal SH, Karickhoff SW, Carreira LA. Prediction of chemical reactivity parameters and physical properties of organic compounds from molecular structure using SPARC. U.S. Environmental Protection Agency Report EPA/600/R-03/030. Washington, DC: U.S. Environmental Protection Agency, 2003.
180. Katritzky AR, Lobanov VS, Karelson M. QSPR: The correlation and quantitative prediction of chemical and physical properties from structure. *Chem Soc Rev* 1995; 24: 279–87.
181. Katritzky AR, Mu L, Lobanov VS, et al. Correlation of boiling points with molecular structure. 1: A training set of 298 diverse organics and a test set of 9 simple inorganics. *J Phys Chem* 1996; 100: 10400–7.
182. Katritzky AR, Ignatchenko ES, Barcock RA, et al. Prediction of gas chromatographic retention times and response factors using a general qualitative structure-property relationships treatment. *Anal Chem* 1994; 66: 1799–1807.
183. Karelson M, Lobanov VS, Katritzky AR. Quantum-chemical descriptors in QSAR/QSPR studies. *Chem Rev* 1996; 96: 1027–1043.
184. Cappelli A, Anzini M, Vomero S, et al. Novel potent and selective central 5-HT₁ receptor ligands provided with different intrinsic efficacy: 1. Mapping the central 5-HT₃ receptor binding site by arylpiperazine derivatives. *J Med Chem* 1998; 41: 728–41.
185. Menziani MC, DeBenedetti PG, Karelson M. Theoretical descriptors in quantitative structure-affinity and selectivity relationship study of potent N4-substituted arylpiperazine 5HT_{1A} receptor antagonists. *Bioorg Med Chem* 1998; 6: 535–50.
186. Miller MD, Yourtee DM, Glaros AG, et al. Quantum mechanical structure-activity relationship analyses for skin sensitization. *J Chem Inf Model* 2005; 45: 924–9.
187. Schultz TW, Cronin MTD. Essential and desirable characteristics of ecotoxicity quantitative structure-activity relationships. *Environ Toxicol Chem* 2003; 22: 599–607.
188. Mazzatorta P, Smiesko M, Piparo EL, Benfenati E. QSAR model for predicting pesticide aquatic toxicity. *J Chem Inf Model* 2005; 45: 1767–74.
189. Schaeffer J, Burch N, Björnsson Y, et al. Checkers is solved. *Science* 2007; 317: 1518–22.
190. Corey EJ. General methods for the construction of complex molecules. *Pure Appl Chem* 1967; 14: 19–37.
191. Corey EJ, Wipke WT. Computer-assisted design of complex organic syntheses. *Science* 1969; 166: 178–92.
192. Corey EJ, Long AK. Computer-assisted synthetic analysis. Performance of long-range strategies for stereoselective olefin synthesis. *J Org Chem* 1978; 43: 2208–16.
193. Corey EJ, Long AK, Greene TW, et al. Computer-assisted synthetic analysis. Selection of protective groups for multistep organic syntheses. *J Org Chem* 1985; 50: 1920–7.
194. Corey EJ, Johnson AP, Long AK. Computer-assisted synthetic analysis. Techniques for efficient long-range retrosynthetic searches applied to the Robinson annulation process. *J Org Chem* 1980; 45: 2051–7.
195. Corey EJ, Jorgensen WL. Computer-assisted synthetic analysis. Generation of synthetic sequences involving sequential functional group interchanges. *J Am Chem Soc* 1976; 98: 203–9.
196. Ugi I, Bauer J, Bley K, et al. Computer-supported chemical calculating, thinking and inventing. *LaborPraxis (Lab 2000)*, 1992: 170–8.
197. Gasteiger J, Clemens J. EROS, a computer program for generating sequences of reactions. *Top Curr Chem* 1978; 74: 93–126.
198. Gasteiger J, Hondelmann U, Röse P, et al. Computer-assisted prediction of the degradation of chemicals: hydrolysis of amides and benzoylphenylureas. *J Chem Soc - Perkin Transactions* 1995; 2: 193–204.
199. Höllering R, Gasteiger J, Steinhauer L, et al. Simulation of organic reactions: from the degradation of chemicals to combinatorial synthesis. *J Chem Inf Comput Sci* 2000; 40: 482–94.
200. Salatin TD, Jorgensen WL. Computer-assisted mechanistic evaluation of organic reactions: 1. Overview. *J Org Chem* 1980; 45: 2043–51.
201. Jorgensen WL, Laird ER, Gushurst AJ, et al. CAMEO: a program for the logical prediction of the products of organic reactions. *Pure Appl Chem* 1990; 62: 1921–32.

202. Fleischer JM, Gushurst AJ, Jorgensen WL. Computer-assisted mechanistic evaluation of organic reactions. 26: Diastereoselective additions: Cram's Rule. *J Org Chem* 1995; 60: 490–8.
203. <http://www.symyx.com/products/databases/>
204. <http://www.cas.org/expertise/cascontent/casreact.html>
205. Gothe SA, Helson HE, Hodaverdis I, et al. Computer-assisted mechanistic evaluation of organic reactions. 22. The generation and use of three-dimensional structures. *J Org Chem* 1993; 58: 5081–94.
206. Baertschi SW, Alsante KM. Stress testing: The chemistry of drug degradation. Chapter 3. In: Baertschi SW, ed. *Pharmaceutical Stress Testing: Predicting Drug Degradation*. Boca Raton, Florida: Taylor and Francis, 2005: 51–140.
207. <http://www.cambridgesoft.com/software/ChemOffice/>
208. Pole DL, Ando HY, Murphy ST. Prediction of drug degradants using DELPHI: an expert system for focusing knowledge. *Mol Pharmaceutics* 2007; 4: 539–49.
209. Socorro IM, Goodman JM. The ROBIA program for predicting organic reactivity. *J Chem Inf Model* 2006; 46: 606–14.
210. Testa B, Balmat A-L, Long A. Predicting drug metabolism: Concepts and challenges. *Pure Appl Chem* 2004; 76: 907–14.
211. Balmat A-L, Judson P, Long A, Testa B. Predicting drug metabolism-an evaluation of the expert system METEOR. *Chem Biodiversity* 2005; 2: 872–84.
212. Judson PN. Using computer reasoning about qualitative and quantitative information to predict metabolism and toxicity. Chapter 24. In: Testa B, Kramer SD, Wunderli-Allespach H, Folkers G, eds. *Pharmacokinetic Profiling in Drug Research: Biological, Physicochemical and Computational Strategies*. Weinheim, Germany: Wiley-VCH, 2006: 417–29.
213. Lhasa, Ltd., 22–23 Blenheim Terrace, Woodhouse Lane, Leeds LS2 9HD, United Kingdom. <https://www.lhasalimited.org/>
214. Judson PN, Vessey JD. A comprehensive approach to argumentation. *J Chem Inf Comput Sci* 2003; 43: 1356–63.
215. Green N, Judson PN, Langowski JJ, et al. Knowledge-based expert systems for toxicity and metabolism prediction: DEREK, StAR and METEOR. *SAR QSAR Environ Res* 1999; 10: 299–314.
216. Button WG, Judson PN, Long A, et al. Using absolute and relative reasoning in the prediction of potential metabolism of xenobiotics. *J Chem Inf Comput Sci* 2003; 43: 1371–7.
217. Judson PN, Marchant CA, Vessey JD. Using argumentation for absolute reasoning about the potential toxicity of chemicals. *J Chem Inf Comput Sci* 2003; 43: 1364–70.
218. Hou BK, Wackett LP, Ellis LBM. Microbial pathway prediction: a functional group approach. *J Chem Inf Comput Sci* 2003; 43: 1051–7.
219. Hou BK, Ellis LBM, Wackett LP. Encoding microbial metabolic logic: predicting biodegradation. *J Indus Microbiol Biotech* 2004; 31: 261–72.
220. Ellis LBM, Rowe D, Wackett LP. The University of Minnesota Biocatalysis/Biodegradation Database: the first decade. *Nucleic Acids Res* 2006; 34: D517–21.
221. <http://umbdd.msi.umn.edu/predict/aboutPPS.html>
222. https://www.lhasalimited.org/zeneth/about_zeneth/
223. Snyder RD, Pearl GS, Mandakas G, et al. Assessment of the sensitivity of the computational programs DEREK, TOPKAT, and MCASE in the prediction of the genotoxicity of pharmaceutical molecules. *Environ Mol Mutagenesis* 2004; 43: 143–58.
224. <http://www.multicase.com/products/prod01.htm>
225. <http://accelrys.com/products/discovery-studio/predictive-toxicology.html>
226. Gombar VK, Enslein K. Use of predictive toxicology in the design of new chemicals. In: Reynolds CH, Holloway MK, Cox HK, eds. *Computer-Aided Molecular Design: Applications in Agrochemicals, Materials, and Pharmaceuticals*. Washington, DC: American Chemical Society; 1995: 236–49.
227. Benigni R. Computational prediction of drug toxicity: The case of mutagenicity and carcinogenicity. *Drug Discovery Today Technol* 2004; 1: 457–63.
228. <http://www.ccl.net/>
229. Kieffer J, Bremond E, Lienard P, et al. In silico assessment of drug substances chemical stability. *J Mol Struct: THEOCHEM* 2010; 954: 75–9.
230. Sharp TR. Calculated carbon-hydrogen bond dissociation enthalpies for predicting oxidative susceptibility of drug substance molecules. *Int J Pharmaceutics* 2011; in press. doi:10.1016/j.ijpharm.2011.04.063

21 | Automation in conducting stress testing and excipient compatibility studies

Eric Carlson, Patrick J. Jansen, and Christopher Foti

INTRODUCTION

Pharmaceutical and biotechnology companies are placing increasing pressure on their development to complete more work with less resources. These companies want to identify and solve issues with compounds at an earlier stage to minimize development costs and time (1). Additionally, quality by design (QbD) initiatives are resulting in more fundamental studies on drug products entering late-stage development in order to understand and quantify the robustness of process conditions. For those activities that are routine and repeated frequently, the use of automation can significantly improve efficiency, throughput, and impact productivity. Stress-testing studies (also generally known as forced degradation studies) yield data that are essential to understanding the degradation chemistry of drug molecules that are in the development phase. These studies are logical places to consider automation since all drug compounds proceed through essentially the same experimental workflow to identify conditions that lead to degradation and to identify degradation products.

There are many examples of various levels of automation being employed as part of a stress-testing program. Fermier et al. (2) have described an instrument designed to expose a multitude of materials to a wide array of stress conditions in parallel, but it does not integrate the collection and processing of analytical data. As a result, it automates only a portion of the overall workflow. Sims et al. (3) have described an approach using online high-performance liquid chromatography (HPLC) analysis to automate and analyze multiple degradation experiments; however, they do not integrate the design or sample generation into their approach. Carlson et al. (4) have described an approach using automation and a comprehensive informatics integration package to perform stress testing of small molecule formulation variants. Each of these examples involves a different part of the drug development process and degree of automation. Automation itself can have an impact across the development process, but there are unique advantages and considerations to various options at different stages of development.

In this chapter, we will review various automation approaches and some general considerations when moving from traditional manual processes to automated processes. We will also provide three detailed case studies where automation is being used to affect a current stress-testing program.

AUTOMATION APPROACHES AND CONSIDERATIONS

Sample Preparation and Processing

Since their introduction in the 1980s, automated liquid handlers (ALH) have found their place in laboratories involved in drug discovery and pharmaceutical development. ALH vary with type and complexity and are offered by a range of vendors (e.g., Beckman Coulter, Inc., Caliper Life Science, Inc., Leap Technologies, Inc., Gilson, Inc., Hamilton, Perkin Elmer, Inc., Tecan, Inc., Thermo Scientific, Inc., Freeslate, Inc.) (5). For automating stress-testing studies, simple liquid platforms such as the PAL autosampler offered by LEAP Technologies (www.leaptec.com) or the Gilson 215 offered by Gilson Inc. (www.gilson.com) may be appropriate when looking to provide automation to solution state studies. Merck has presented a system based on a Leap Technologies PAL system for solution station stress-testing studies (6). For more comprehensive automation or when dealing with solid-state samples, slurries, the need to adjust pH, or biological formulations, a platform that offers additional unit operations such as solids dosing, sample weighing, viscous handling, automated capping, and pH monitoring may be appropriate. An example of such a system is the Core Module offered by Freeslate, Inc. (www.freeslate.com).

Stress-testing studies also typically include processing or conditioning samples with controlled environmental exposure such as elevated temperature, temperature cycling, controlled humidity, controlled light exposure, and even controlled mechanical stress. As with sample preparation, there is a spectrum of available choices to use in an automated stress-testing workflow. Many companies have in-house automation groups that are able to customize their own chambers or solutions. There are many commercially available options that can also be incorporated into an automated workflow. For example, the reaction racks offered by ReactArray (www.reactarray.com) can be integrated with a Gilson 215 robot for controlled heating and sampling of an array of samples, as described below in section “A Semiautomated Solution State Stress Testing System.” Liconic offers a range of robotically accessible environmental incubators and chambers (www.liconic.li) that control temperature and humidity and that serve up SBS-format arrays (7), as described in sections “A Fully Automated Solid and Solution State Stress-Testing System” and “Automation for Stress-Testing Biopharmaceutical Formulations” below. Some robotic stations also offer on deck heating and sample processing. Processing and conditioning of samples can vary from simple aluminum block designs used as a reaction arrays, offering limited stirring and temperature control, to the latest generation of array-based lab reactors designed for parallel synthesis and examples of automated systems for stress testing include a range of such sample processing stations as exemplified by the case studies in this chapter.

Sample Analysis

Samples generated using an automated system will typically be analyzed using some type of chromatographic procedure. As is discussed in chapter 4 of this book (Stress Testing: Analytical considerations), HPLC with ultraviolet (UV) detection is often the method of choice. There are two main approaches to development of methods used for analysis of stressed samples. The first approach involves the use of generic HPLC methods that can be applied to a broad range of compounds. The benefit of this approach is that a separate HPLC method will not need to be developed for each compound undergoing stress-testing studies. Generic methods will typically utilize a C8 or C18 reversed-phase column with gradient elution in order to be able to retain and separate analytes that span a wide polarity range. If the compounds being tested contain a chromophore, then the use of a photodiode-array detector (PDA) is recommended so that chromatograms can be extracted at multiple wavelengths since degradation products may not absorb at the same wavelength as the parent compound. The use of a PDA also enables the examination of the homogeneity of the parent peak. The UV-homogeneity (or lack thereof) of a chromatographic peak can be useful for discovering degradation products which co-elute with the parent. The pH of the mobile phase can have a dramatic effect on the chromatographic separation of compounds which contain an ionizable functional group. A detailed discussion on choice of mobile phase is beyond the scope of this chapter, but some general recommendations can be made. In general, suppression of ionization will tend to cause analytes to be retained on a reversed-phase column longer resulting in better separations. For example, a low pH mobile phase (e.g., trifluoroacetic acid, acetic acid, formic acid, and low pH phosphate buffer) will suppress ionization of compounds containing a carboxylate group while a neutral to high pH mobile phase (e.g., neutral pH phosphate, bicarbonate, and ammonium hydroxide) will suppress ionization of basic amines.

Alternatively, chromatographic methods for automated stress-testing studies can also be developed via rapid LC screening as a starting point (8–11). This is a more rigorous scientific approach that will be more time consuming than utilizing generic methodology, but allows the analytical scientist to evaluate selectivity and the impact of pH and stationary phase on chromatographic performance. Key attributes of the chromatographic methods for online analysis are speed for detailed kinetic analysis, resolution, and detection that is orthogonal to UV for evaluation of all potentially relevant degradants. This may include a variety of detection techniques such as evaporative light scattering detection (ELSD), charged aerosol detection (CAD),

fluorescence, electrochemical, or mass spectroscopy (MS) detection. Peak purity information of the main component may also be obtained using PDA or MS detection. The chemical and physical properties of the degradants may necessitate the use of these alternate detection techniques which can impose limitations on mobile phase and wavelength selection and complicates data processing and storage when examining multiple degradation conditions.

Informatics

The use of automation will allow the generation of much more data than is typical with manual procedures. The result is that automation likely requires a process or strategy for managing the new influx and amount of data. There are several strategies that range in complexity and in the ability to organize, analyze, and share back data quickly. Generally, systems based on file servers and data repositories are likely to be insufficiently robust or flexible; while they are easy to set up in order to locally warehouse data, it can prove to be challenging to efficiently mine such systems for data to assemble reports. In addition, they are typically not scalable over time.

There are several different types of informatics systems. Many chromatography systems are supported by software that allows one to store and analyze collections of analytical data. Examples include Agilent's Chemstation®, Dionex's Chromeleon®, and Waters' Empower®. These software packages, however, only address a portion of the scientific workflow and are generally focused on the manipulation of the analytical data. Software such as Laboratory Execution and Analysis (LEA) offered by Freeslate and Accelrys, Inc. is designed to enable the whole experimental workflow for scientists—from design, to execution, to analysis, through reporting. LEA integrates multiple devices, instruments, and analytics while aggregating the data. Rather than passing worksheets to an instrument and collecting results, LEA offers full automation of the process including remote instrument control from a single-user interface and the ability to query, analyze, and report on the raw data from a variety of data types.

Finally, to fit within a company's process or to support QbD initiatives, one may want also leverage an enterprise-wide informatics system. The software packages can include Laboratory Information Management Systems (LIMS), Scientific Data Management Systems (SDMS), and Electronic Laboratory Notebooks (ELN). The focus and use of packages, such as LEA, LIMS, SDMS, and ELNs, vary. LIMS are sample management and tracking systems (i.e., studies, projects, batch/lot, plates, and task assignments) with additional functions for tracking laboratory assets, including purchase and calibration dates. SDMS are designed for archiving both document and instrument data and are used to provide automated capture and retrieval of raw data files instrument reports, metadata extraction, cataloging, storing, and archiving reports in a software-neutral format that does not require the original source software to view the reports. ELNs are a replacement for paper-based notebooks for specifying experiment content and context. LIMS, SDMS, ELN, and LEA all play important roles within the scientific workflow. Generally speaking, LIMS, SDMS, and ELN each cover portions of the scientific workflow, whereas LEA, designed for experiment-centric workflows, brings together all four steps and allowing scientists to bring together data from design to processing, to analytics from a variety of sources in a single environment in order to make decisions (as shown in Fig. 1). A system offering LEA's overarching role in pulling together the full workflow becomes more powerful as the fraction of automated equipment in laboratories increases as shown in some of the examples later in this chapter.

CASE STUDIES

A Semiautomated Solution State Stress-Testing System

In this section, a system in use at Pfizer's Development Labs in Groton, CT is described to enable semiautomated solution state stress testing (the ReactArray system). Anachem integrates their software control with a Gilson 215SW liquid handler and a STEM reaction block (Fig. 2). The ALH is a small footprint one arm X, Y, Z-robot on a platform equipped with a dual-syringe pump capable of volume delivery from 1 μ L to 25 mL per piston stroke, a bed capacity

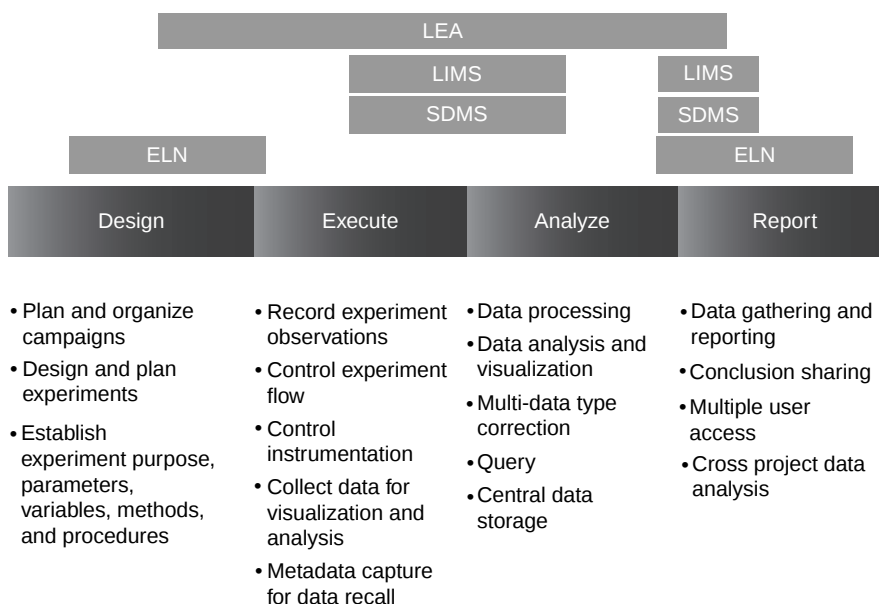


Figure 1 Overview of inter-related informatics packages enabling the scientific workflow.

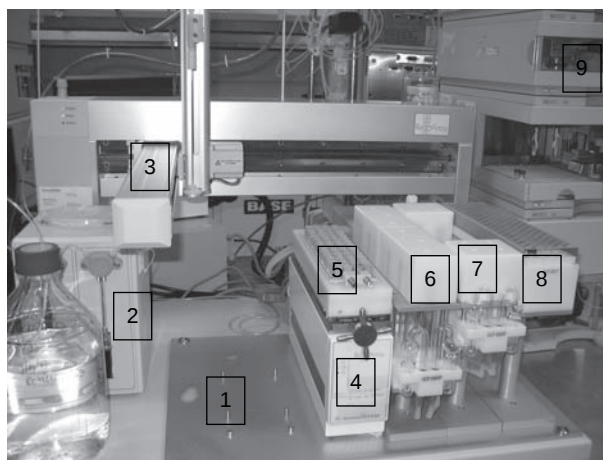


Figure 2 ReactArray semiautomatic solution state stress-testing system: **1.** Gilson 215SW Liquid Handler Platform; **2.** Gilson Dual Syringe Pump; **3.** Robotic X, Y, Z arm with septum piercing probe; **4.** STEM RS 12 Reaction Block; **5.** Reflux condenser; **6.** 40 mL reagent rack; **7.** 130 mL reagent rack; **8.** Analytical Rack. **9.** HPLC.

to hold up to four custom racks which may consist of either variable volume reagent racks, a well-plate rack to handle samples delivered in this format or analytical racks which contain standard HPLC vials. The platform is capable of a number of liquid handling tasks from reagent addition, analytical sample dilution, and preparation as well as optional online analysis by HPLC. Sample is injected on the HPLC via the two-position six-port switching module for sample loading and full-loop sample injection. The sample loop size is fixed so selection of the loop volume should best suit the LC system and or method developed. Typical detectors can vary from diode array UV/Vis, mass spectrometry, and charged aerosol detectors and will be

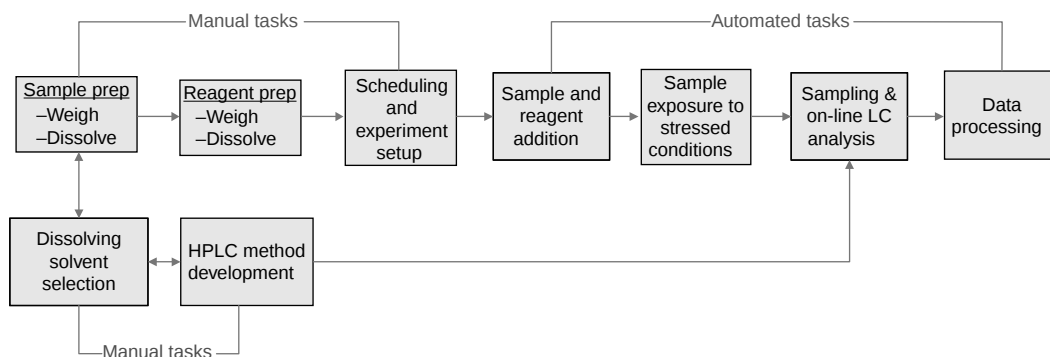


Figure 3 Manual and automated processes of Pfizer's early stress-testing workflow.

dependent on the respective analyte properties. Interface with the Agilent LC occurs by contact closure communication via a 6890 universal remote cable to the G1315A diode array detector. Communication between the Agilent MSD occurs through an RS232 remote cable to the pump. The STEM RS 12 Reaction block with refluxing head is positioned on the liquid handler platform in one of the four rack positions and has 12 temperature zones (-30°C – 150°C) with 48 micro reaction vessels capable of handling volumes from $250\ \mu\text{L}$ to $2.5\ \text{mL}$.

In order to achieve successful predesigned solution stress conditions, it is imperative that reliable liquid transfer of reagents occur to ensure the proper amounts of key reagents. This aspect was demonstrated by programming the ALH to prepare and deliver caffeine samples with concentrations ranging from 0.01 to $0.1\ \text{mg/mL}$. The caffeine stock solution volumes ranged from 50 to $500\ \mu\text{L}$. The final analysis of these samples by HPLC resulted in an $R_2 = 0.998$, thus confirming excellent accuracy of the sample delivery.

Description of Workflow and Process

Three laboratory processes that were implemented using the automated ReactArray platform included early stress-testing studies, structure activity relationship (SAR) assessments, and pH stability assessments since they are qualitative, non-GMP, involve repetitive operations that are tedious, time consuming, and are ideal programs for high throughput. These early stress-testing studies are designed to elucidate the degradation pathways of a drug candidate, identify degradation products, and to assist in the development of stability indicating methodology. These studies also allow the generation of experimental data in an expedited manner that will ultimately accelerate predictive stability assessments. The use of preprogrammed automated protocols enable the user to perform purposeful degradation work designed to yield SAR information from series of chemotypes to influence new chemical entity selection. Additionally, the pH stability assessments in gastric (pH 1.2) and intestinal pH (6.8) solution matrices without enzymes at 37°C are needed to support of formulation development of oral dosage forms. The workflow schematic designed around these processes is shown in Figure 3. The manual tasks consist of design and scheduling of experiments, reagent and API stock solution preparation, solvent and HPLC method selection and programming. Tasks that are carried out by the automated ReactArray workstation are reagent addition, sample addition, mixing, heating, sampling, and injection for online analysis by liquid chromatography. HPLC analysis is typically accomplished using an Agilent 1100 LC with diode array and mass spectrometric detection. The data processing is performed using an in-house designed Agilent Chemstation® macro which exports the integrated data file with selected chromatographic parameters into a csv file. The csv file is further manipulated and all data formatted in an excel format for reporting and graphical representation where necessary.

Reacting vessel	Temp.	Degradation condition	Samples prepared at							Samples prepared at							Samples prepared at						
			0 hr							12 hr							18 hr						
RV01		Blank	1,2,3																				
RV02	60°C	API		4												16							
RV03	60°C	ACVA			5												17						
RV04	60°C	API + 30 mol% ACVA				6				11							18						
RV05	30°C	API					7				12							19					
RV06	30°C	API + 0.3% H ₂ O ₂						8				13								20			
RV07	30°C	API + 1N HCl						9					14								21		
RV08	30°C	API + 0.8N KOH							10					15									22

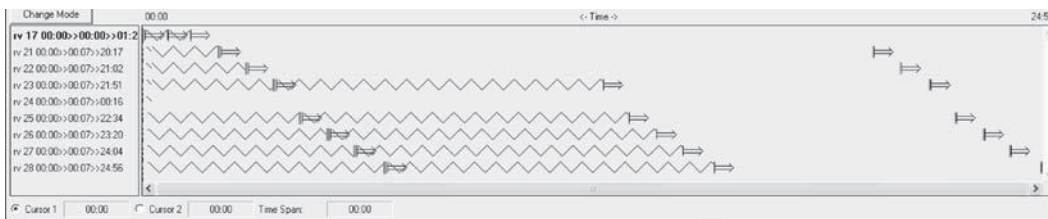


Figure 4 ReactArray conditions and scheduling window for purposeful degradation studies.

Prior to initiating any automation experiment, it is important to select an inert cosolvent that will ensure effective solubility of the reagents and API throughout the process. Sample precipitation may be unavoidable during some kinetic points depending on the sample interaction with the stressing reagents. Another key point to consider is cosolvent compatibility with the HPLC conditions. The desirable properties of the organic cosolvents used in nonautomated forced degradation are discussed in the literature and are equally important in the automated approach (12,13). To minimize aqueous and organic solvent usage and for effective online analysis, the ideal chromatographic methodology typically consists of mobile phases that are MS friendly and that utilize 100 mm × 3.0 mm ID columns and 3 µm particles with a method run time of between 20 and 35 minutes. The use of 3 mm ID columns generally ensures flow rates < 1.0 mL/minute. The LC methodology may be generic or derived from LC screening.

Automated Stress-Testing Studies

The degradation conditions and kinetic time points in addition to the schedule of tasks as illustrated by the ReactArray software for an automated stress-testing study is shown in Figure 4. Eight 100 mL reaction vessels are used primarily to deliver the acid/base, radical initiator, peroxide, and dissolving solvent solutions for the hydrolysis, radical, and peroxide oxidation as well as the respective control conditions. Incorporation of key design parameters to support early development work include requiring a minimum of 5–10 mg of API to conduct a study, thus using a prepared 10 mL stock at nominal HPLC assay concentration to minimize sample preparation tasks and to facilitate analysis completion over a 24 hours cycle time. Throughout the typical 18 hours forced degradation reaction sequence, a total of 22 sample aliquots are removed from the reaction vessels and analyzed. All samples are analyzed at the initial, 9 and 18 hours time points for each degradation condition and compared to respective controls. Additionally, the 18 hours samples are collected in 2 mL HPLC vials for further evaluation by the analytical scientist. Activation temperature is a key parameter in driving the free radical oxidative forced degradation experiments; therefore, 60°C is required when utilizing 4'-azobis (4-cyanovaleric acid) (ACVA) as the radical initiator. The remaining degradation studies are conducted at 30°C.

The chromatograms in Figure 5 are the online acid/base results obtained using the ReactArray workstation for an API stress-testing study in early development. The key predictive sample set for this example is acid/base hydrolysis where complete conversion to the primary degradant occurs after 9 hours of aqueous potassium hydroxide hydrolysis. Furthermore, the

interface with mass spectrometry showed the identified as the amide hydrolysis product. By using mass spectrometry as an online tool, the analytical scientist is able to track degradants and evaluate main band peak purity. This example also illustrates the ReactArray's capability for preparation of degradants as part of a stress-testing study.

Automated pH Stability Assessments

Assessment of pH stability under simulated conditions in the stomach and intestine when combined with solubility and other preformulation data can be used as a development tool to aid in the selection of a formulation and/or different properties of a compound. Sample solutions are prepared at the HPLC assay concentration (i.e., 0.1–1 mg/mL) in a solution matrix of pH 1 and 6.8 (Table 1) and heated to 37°C. Samples with low aqueous solubility are prepared with the appropriate cosolvent. The final solution compositions are consistent with the USP for gastric and intestinal conditions without enzymes. Withdrawal of sample aliquots for online HPLC analysis occurs approximately every 2 hours with the majority of the kinetic points within 12 hours and completing in 24 hours. As seen in Figure 6, gastric conditions at a physiologically relevant temperature yielded data indicating that the compound is unstable in acid/gastric conditions versus the API control and pH 6.8 conditions. Evaluation of the LC/MS data showed that the degradants were the amide hydrolysis products of the API.

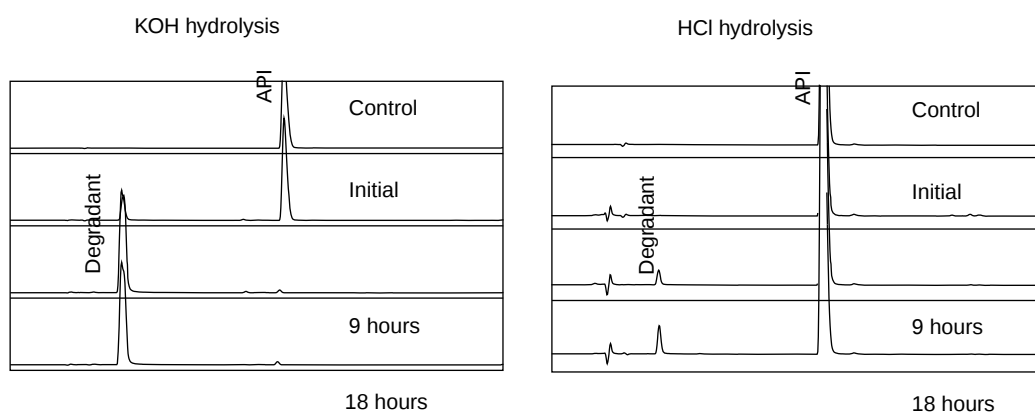


Figure 5 Online HPLC results from KOH and HCl hydrolysis.

Table 1 pH Stability Challenge Conditions and Protocol

Type of Exposure	Challenge Conditions	Kinetics
pH 6.8	67 mM KH_2PO_4 , API sample concentration = 0.1–1 mg/mL	Initial and 2.25 hrs intervals
pH 1.1	0.05 M NaCl, 0.04 M HCl, API sample concentration = 0.1–1 mg/mL	Initial and 2.25 hrs intervals
API control	Dissolving solvent at 37°C, API sample concentration = 0.1–1 mg/mL	Initial and 2.25 hrs intervals

Abbreviation: API, Active pharmaceutical ingredient.

Automated SAR Studies

The following examples illustrate the use of solution semiautomated workstations to evaluate SAR of a series of compounds through differences in azo-bis-cyano-valeric acid (ACVA) oxidation (14) and HCl hydrolysis (Fig. 7). Six compounds were evaluated for oxidative susceptibility and seven compounds were tested for acid hydrolysis susceptibility. The large number of compounds and kinetic points makes these types of experiments very conducive to automation.

Impact and Ease of Implementation

The implementation of these solution semiautomated systems should be determined by capital investment, robustness, and ease of use. From a strictly capital perspective, the systems are a relatively low-cost investment. Pragmatically speaking, the question becomes how elaborate do you design these systems. One may simply use the platform as a sample preparation station and perform all analyses offline. Conversely, interfacing with external devices such as LC and various detection modes introduces not only additional cost but potential component communication issues. Since these platforms may be designed using components from different vendors, finding comprehensive technical support to complete the interfacing presents a new challenge. Most vendors only commit to the operations of their equipment. The Achilles heel of these types of liquid handler systems for the applications cited in this chapter is precipitation of the API which can lead to injector port clogging, reduced sensitivity, and sample carry-over. These issues may be minimized with routine upkeep of the handling system including the implementation of aqueous/solvent wash cycles. Also as important is the maintenance of clean vessel glassware and related hardware. For example, the reflux head needs periodic cleaning to avoid cross-contamination issues and the septa need replacement to minimize injector port clogging. The impact of this semiautomated technology for a variety of stress testing is that tedious, routine, and repetitive tasks are automated, thus enabling the analytical scientist to minimize error and become more efficient.

A Fully Automated Solid and Solution State Stress-Testing System

The Freeslate Forced Degradation System is a custom-built, integrated system designed to carry out stress-testing studies in an automated fashion and is in use at the Eli Lilly Technology

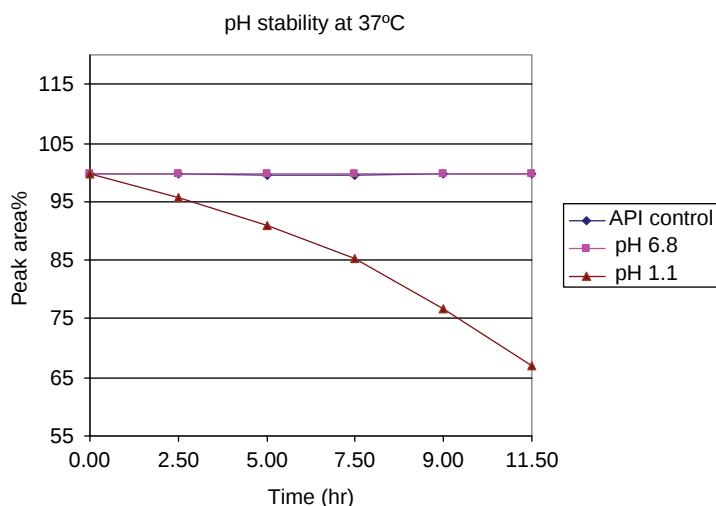


Figure 6 Gastric/intestinal pH buffer study using the ReactArray workstation.

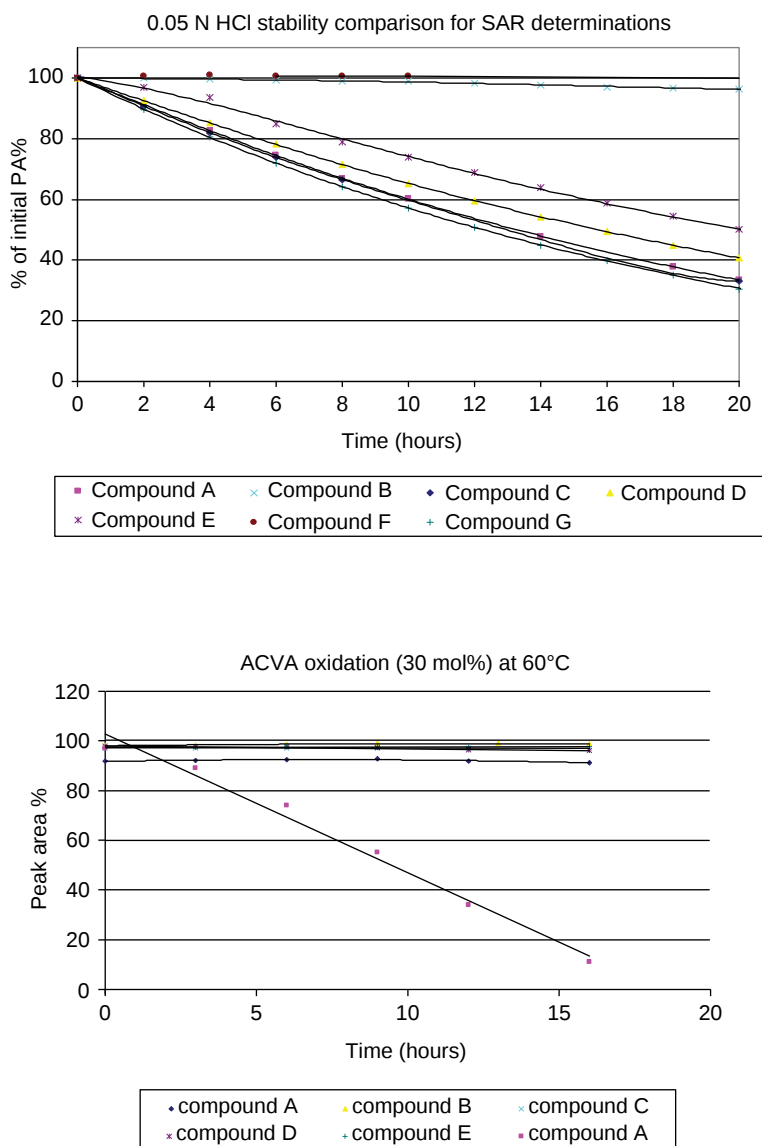


Figure 7 Structure activity relationship (SAR) studies.

Development Center in Indianapolis, IN (15). A visual representation of the system is shown in Figure 8. The system consists of a Freeslate Powderium® powder dispensing robot, a Free-slate Core Module, an articulated plate moving robot, plate stackers, a barcode printer/appliator, environmental chambers, a refrigerated chamber for cold storage of samples, and Waters' Aquity® ultraperformance liquid chromatography (UPLC) instruments. The powder dispensing robot is physically separate from the remainder of the system to increase flexibility since it has many other applications other than dispensing samples for stress-testing studies. Although not physically integrated with the remainder of the system, the powder dispensing robot uses the same software control as the rest of the system, Freeslate's LEA software suite, discussed in section "Informatics." The Core Module is a two-arm liquid, plate, and vial handling robot.

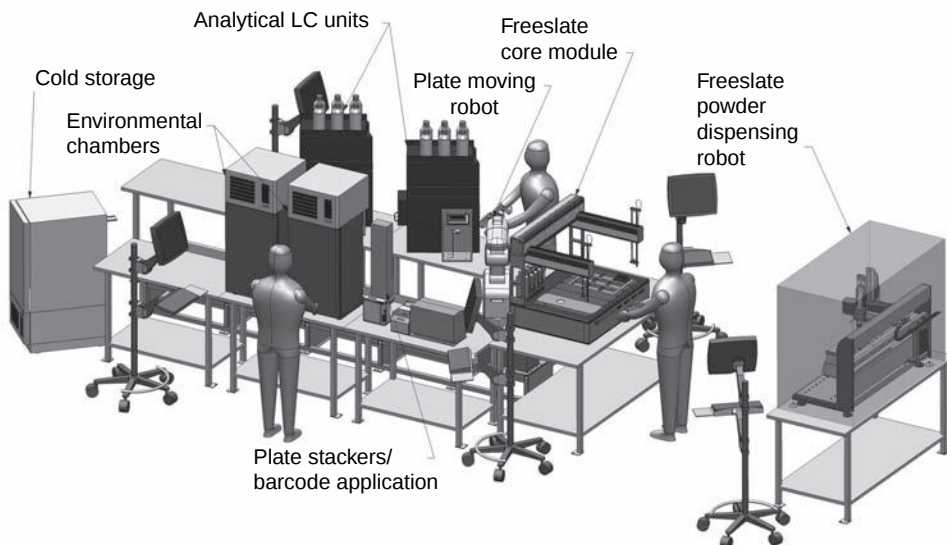


Figure 8 Visual representation of Freeslate Forced Degradation System.

It incorporates a balance capable of accurately weighing individual vials, a camera used to image samples, a pH probe, heated bays with embedded magnetic stirrers, and a vortex unit which is capable of vortexing entire plates. The environmental chambers and cold storage unit are manufactured by Liconic Corporation and contain robotic mechanisms which allow plates to be stored and retrieved in a completely automated fashion. All of the components of the system are computer controlled by Freeslate LEA software.

A typical stress-testing study using this system consists of two types of workflows: a solid-state workflow in which solid samples of the test compound are stressed under thermal/humidity conditions and solution workflows in which solutions of the stressed compounds are stressed under various conditions. A typical solid-state workflow is shown in Figure 9. The first step in this workflow is using the Core Module to accurately weigh the individual 4 mL vials in a 4×6 library plate in order to get their tare weight. The plate is then manually transferred to the powder dispensing robot which dispenses a defined amount of solid material to each vial. The powder dispensing robot contains a low-resolution balance which actively monitors the weight change as powder is dispensed into each vial in order to achieve the approximate target dispense level. Following powder dispense, the plate is manually carried back to the Core Module and the vials reweighed to get the accurate weight of the powder dispensed into each individual vial. The entire plate is then transferred to one of the humidity and temperature-controlled environmental chambers using the plate moving robot. At specified time points, the plate is removed from the environmental chamber and transferred back to the Core Module. Selected vials are then plucked from the library plate and transferred to a storage plate using the vial handling capabilities of the Core Module. The library plate is transferred back to the environmental chamber and the storage plate is transferred to the cold storage unit. This process is repeated until all time points have been completed. After all time points have been moved, the samples are prepared for analysis. The appropriate solvents are added to the vials followed by vortex mixing to ensure dissolution of samples and another sample weighing step

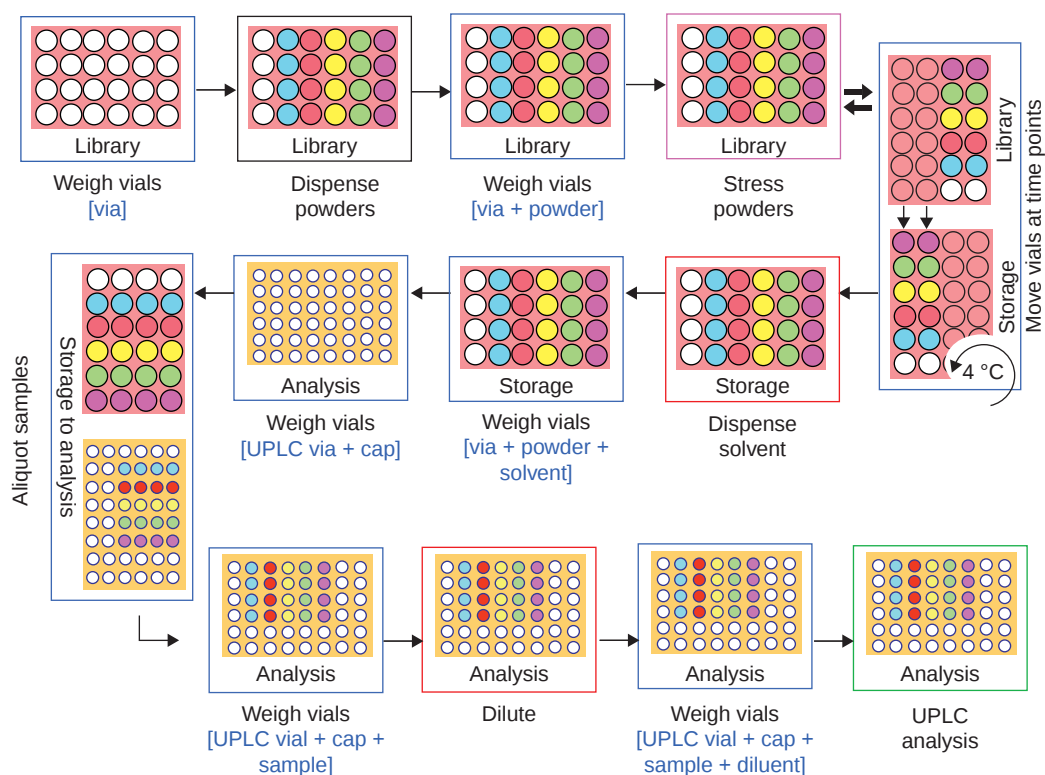


Figure 9 Typical Freeslate Forced Degradation System solid-state workflow.

to accurately determine the exact amount of solvent dispensed. If the solutions are at the appropriate concentration, a portion of the sample is transferred directly to the analysis vials for chromatographic analysis. If a serial dilution step is needed, the analysis vials are individually weighed, the sample transferred, the vials weighed again to confirm amount of sample added, the dilution solvent added, and the individual vials weighed a final time. Gravimetric confirmation of the amount of solvent added at all of the solvent addition steps significantly increases the precision of the sample preparation procedure. The prepared samples are typically analyzed versus unstressed samples (controls) prepared in the same manner.

A typical solution workflow is shown in Figure 10. The first step in this workflow is using the Core Module to accurately weigh the individual 20 mL vials in a 2 × 4 library plate in order to get their tare weight. The plate is then manually transferred to the powder dispensing robot which dispenses a defined amount of solid material to each vial. Following powder dispense, the plate is manually carried back to the Core Module and the vials reweighed to get the accurate weight of the powder dispensed into each individual vial. The appropriate solutions are then added followed by vortex mixing and another weigh step to accurately determine the amount of solvent added. The next step is the weighing of the individual analysis vials followed by the solution fill. After this fill, the vials are weighed again to determine the weight of solution added and then the plate is transferred to the defined storage condition. At specified time points, the plate is removed from its storage condition and transferred back to the Core Module. Selected vials are then plucked from the storage plate and transferred to an analysis plate using the vial handling capabilities of the Core Module. The storage plate is transferred back to the environmental chamber and the analysis plate is transferred to the cold storage unit. This process is repeated until all time points have been completed. After all time points have

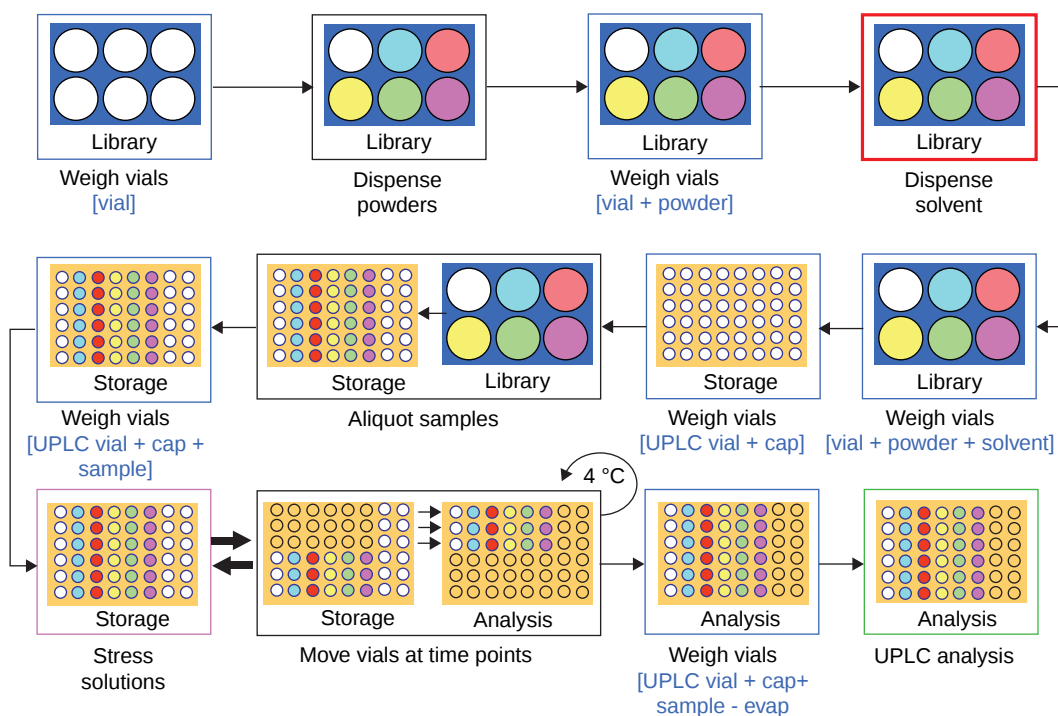


Figure 10 Typical Freeslate Forced Degradation System solution workflow.

been moved, the individual sample vials are weighed again to measure the amount of solution evaporation just prior to chromatographic analysis.

A unique aspect of the Freeslate Forced Degradation System is the ability to track solvent manipulations (addition, serial dilutions, evaporation) gravimetrically. This allows the system to achieve a high degree of accuracy and precision and enables the system to accurately track the loss of the parent compound. Estimates of the precision capabilities of the system were obtained for both the solid-state and solution workflows. The solid-state workflow precision was estimated by preparing and analyzing 18 individual samples using the automated system, and the solution workflow precision was estimated by preparing and analyzing eight individual samples using the automated system. The results are provided in Table 2. The results given in the table are an estimate of the entire system precision including the chromatographic analysis.

Prior to installation of the Freeslate Automated Forced Degradation System, all stress-testing studies were carried out manually using conventional sample preparation techniques. During the 5 year period prior to implementation of the Freeslate system, a total of 20 stress-testing studies supporting late-phase drug candidates were carried out utilizing approximately one full time equivalent. This corresponded to approximately 13 weeks of work per study. The Freeslate Automated Forced Degradation System was designed, assembled, and tested over the course of approximately 1 year. The 6 months following installation were spent testing, training, troubleshooting, and qualifying the system. An approximate doubling of efficiency over the manual process was realized in the first year of full utilization of the automated system. Further refinements to the system have resulted in a three times gain in efficiency in subsequent years when compared to the manual process. In addition, these efficiency gains have significantly increased the stress-testing study capacity which has enabled the studies to be pushed further back on the development timeline without significantly increasing costs and

Table 2 Solid-State Workflow Precision

Concentration (mg/mL)	Response	Relative Response Factor	% Deviation from Mean
0.479	1,151,262	2,405,152	-4.69
0.515	1,330,244	2,583,920	2.40
0.516	1,318,506	2,557,478	1.35
0.520	1,337,288	2,569,776	1.83
0.523	1,322,498	2,526,785	0.13
0.525	1,352,721	2,578,953	2.20
0.527	1,328,689	2,519,926	-0.14
0.530	1,320,616	2,490,578	-1.30
0.537	1,361,273	2,534,283	0.43
0.538	1,368,687	2,546,300	0.90
0.538	1,365,984	2,540,209	0.66
0.542	1,395,068	2,571,960	1.92
0.543	1,376,471	2,533,237	0.39
0.545	1,379,922	2,531,606	0.32
0.546	1,363,269	2,496,257	-1.08
0.546	1,361,920	2,492,195	-1.24
0.548	1,367,133	2,496,498	-1.07
0.560	1,369,989	2,447,486	-3.01
		Standard deviation = 46,519	
		RSD = 1.84%	
Solution Workflow Precision			
0.481	1,183,752	2,463,527	-1.13
0.511	1,286,035	2,514,654	0.92
0.513	1,293,076	2,520,137	1.14
0.522	1,291,166	2,474,818	-0.68
0.522	1,304,094	2,497,961	0.25
0.525	1,316,339	2,505,702	0.56
0.530	1,311,770	2,474,112	-0.71
0.536	1,330,262	2,483,412	-0.34
		Standard deviation = 20,797	
		RSD = 0.83%	

enabled consideration of stress testing of intermediates as well. Conducting stress-testing studies earlier enables identification of potential stability issues sooner, eliminates the expense of redundant studies, is foundational to early phase method development, and is consistent with the concept of building in quality through scientific knowledge and design.

Automation for Stress-Testing Biopharmaceutical Formulations

Large molecule development presents additional challenges as compared to their small molecule counterparts when considering automated methods of stress testing. In particular, large molecule stability is not just measured by changes in primary structure (i.e., oxidative and deamidative degradation), but in secondary and tertiary structure changes as well (16). In addition, irreversible aggregation and interactions with the final container-closure system for the desired drug product are issues not typically evaluated at early development stages for small molecules, but can be essential for the development of a biological formulation as is discussed in chapter 23 of this book.

The result is the need for more complex and orthogonal analytical techniques to map a variety of the chemical and physical instabilities that may result during formulation

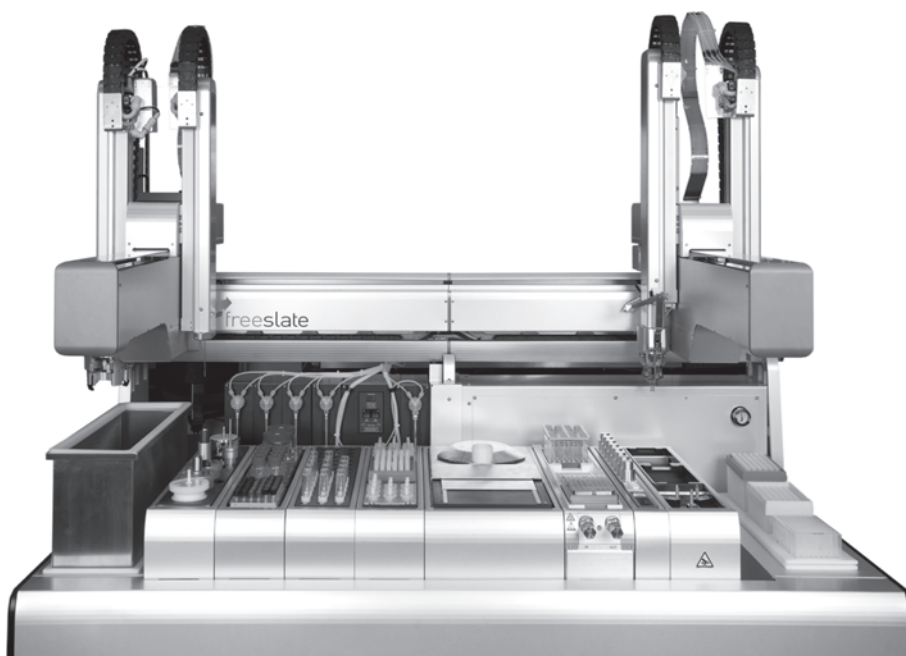


Figure 11 Freeslate Core Module for automated sample preparation including liquid dispense, viscous dispense, mass tracking, pH measurement, digital image capture, sample vortexing, sample heating and cooling, and sample capping and uncapping.

development for large molecules. Hence, automated workflows must generally integrate with a number of analytical devices, each having its own sample preparation requirements. In addition, due to the diversity of analytical data types and potential diversity in the study design, it becomes nearly imperative to have an informatics platform that can handle and allow a scientist to make conclusions on large analytical datasets. Furthermore, there is generally limited material in early phase biologics development, and thus, there is a need for automated processes that can consistently handle small quantities of material.

In this section, examples of automated biologic formulation stability testing systems are described that are in place at Freeslate in Sunnyvale, CA. In the first example, a Freeslate Core Module (shown in Fig. 11) is used as an automation station to prepare, work-up, and analyze samples, and LEA software is used as comprehensive informatics integration package to tie together additional data from dynamic light scattering (DLS) and from HPLC stations that are not physically integrated into the system.

Such a station is used in early development studies to explore formulation composition for stability. For example, proprietary protein Y was formulated with several combinations three different surfactants (polysorbate 20—P20, polysorbate—P80, and poloxamer 188—F68) and six different total surfactant levels to achieve 42 unique formulation and series of controls to find the optimal formulation for protein Y. The library design is shown below in a screen shot from LEA's Library Studio software package (Fig. 12).

The above library design was saved to a database, and LEA software connected to the Core Module in Figure 11 was able to automatically formulate the library by loading and executing the design recipe generated with the LEA suite of software. A well plate of 4 mL vials was robotically tarred. The system buffer and each surfactant as added to each well and the transferred weight was verified by the on-deck balance. Protein was added in solution, and samples were mixed by vortexing the plate.

To analyze the formulations, each sample was analyzed by automated on-deck digital imaging (on-deck), off-deck dynamic light scattering (DLS), and off-deck size-exclusion chromatography (SEC). To gather the images, the Core Module used a vial gripper to move each sample in front of a digital camera and captured a digital image (Fig. 13). The plate was manually loaded into a Wyatt DLS instrument. Using LEA software, a library identifier was entered, and data collected and stored back to the database along with the library design and the digital images (Fig. 14). Finally, the Core Module was used to create dilutions of the protein Y formulations for size exclusion chromatography (SEC) analysis. The dilution plate was manually loaded into an Agilent 1200 HPLC configure for SEC analysis. The HPLC was integrated with LEA software, and in addition to being able to load up dilution factors and capture the HPLC data, LEA also allowed decisions on which samples to be analyzed with SEC to be made based on the subsequent analysis. Because the SEC method is lengthy (30 minutes isocratic method with a mobile phase comprising 0.2M potassium phosphate, 0.25M KCl, pH 7.0, run at 0.5 mL/min at 25°C using a Tosoh TSK-Gel G3000SWXL column, 7.8 × 300 mm, 5 µm), and one generally does not want to flow precipitate through the columns, additional efficiency was

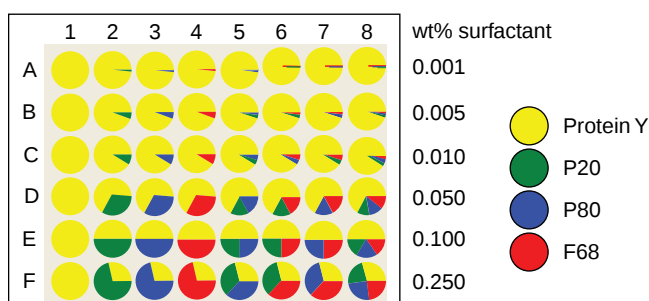


Figure 12 Graphical representation of library design. Weight percent of components shown with the aqueous buffer solution suppressed.

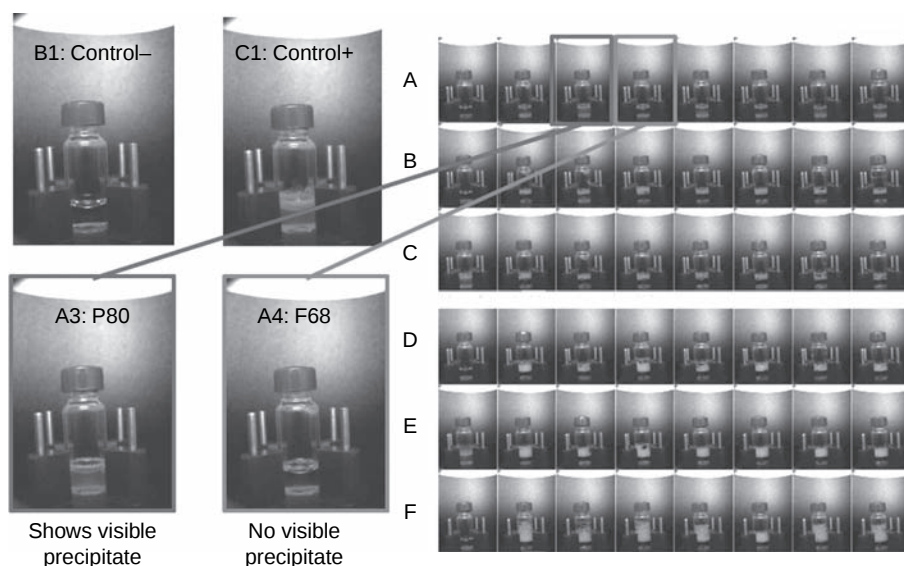


Figure 13 Automated digital image analysis of library of protein Y liquid formulation samples.

gained by using both digital imaging and DLS to identify that substantial amounts of protein Y had precipitated in row A of the library. Thus, only SEC data of wells of interest were collected (Fig. 15).

In order to demonstrate a comprehensively automated stress-testing system for large molecule formulation development, Freeslate has also put in place a fully automated system to test for protein formulation stability and excipient compatibility. The system has many features in common with the system in operation at Eli Lilly described in the section “A Fully Automated Solid and Solution State Stress-Testing System.” This automated bioformulations workflow

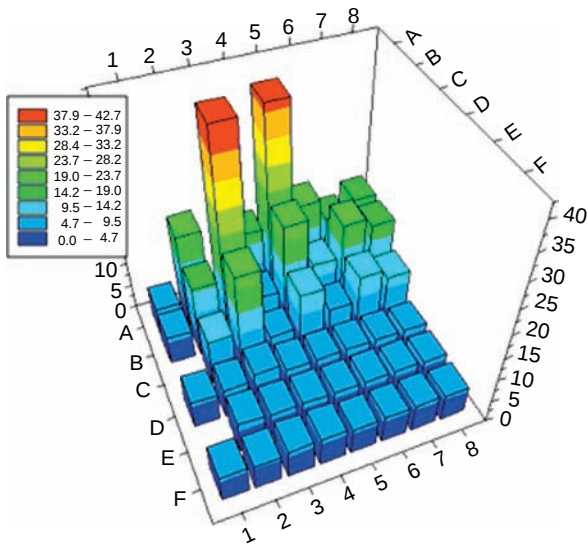


Figure 14 Measured hydrodynamic radius (nm) of samples from library of protein Y liquid formulation samples based on automated DLS measurements.

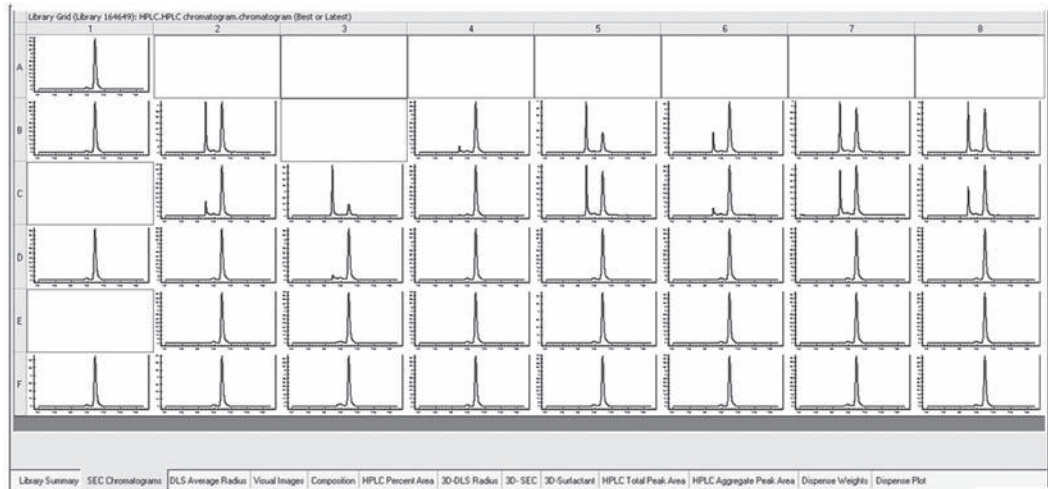


Figure 15 Screenshot from LEA showing the SEC chromatograms of library of protein Y liquid formulation samples. Blank wells were intentionally not analyzed by SEC based in previous digital image and DLS analysis.

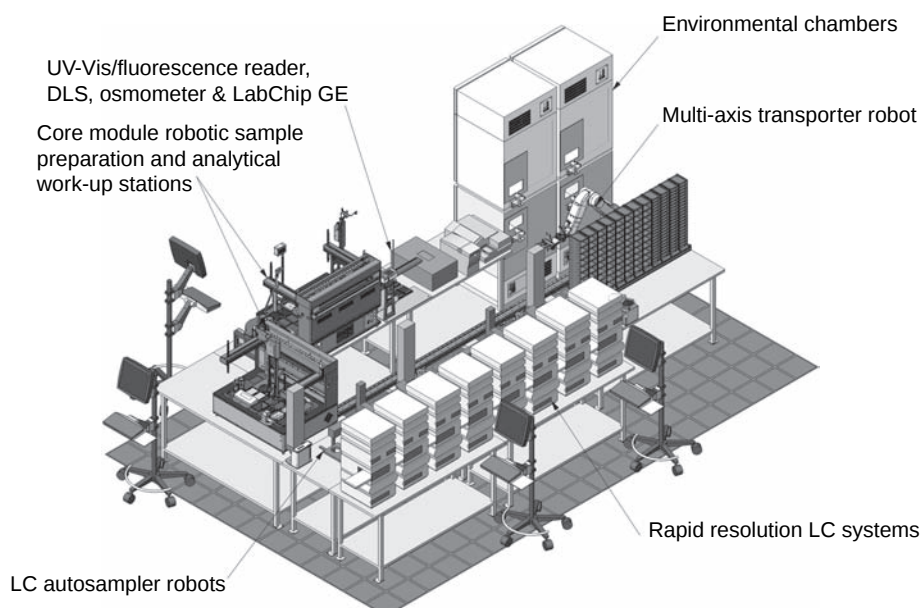


Figure 16 Fully automated biopharmaceutical formulation stability testing workflow.

also has a several sample preparation robots integrated physically via a six-axis robotic arm on a linear track with changeable end effectors to environmental stress chambers, various analytics, and analytical sample loading robotics, all under the control of an integrated LEA software platform that allows for automated stress testing of matrix-based formulation experimental designs. As is required for biological formulations, this system integrates with and provides for the automated sample preparation of samples for several diverse analytical techniques such as SEC, ion-chromatography, osmolality, pH, Lab-chip electrophoresis (i.e., SDS-PAGE), dynamic light scattering, and UV-Vis assays. The system is designed to modularly accommodate samples filled in final container-closure systems enclosure or to use arrays of micro-quantities of formulation for early exploration when materials are limited (Fig. 16).

This workflow assigns a library ID (and associated barcode) to each rack of samples, with each rack representing a stress condition and timepoint for the stability study. The workflow physically integrates and automates the storage of racks in temperature-controlled chambers, scheduling of samples for analytical sample preparations, automation of the analytical sample preparations, and movement of analytical samples to the autosamplers of the relevant analytics. In certain cases, some stress conditions may be physically offline from the automated workflow, yet still integrated from an informatics standpoint (for example, mechanical stresses, photostability stresses, etc.). This modularity is made possible by the nature of the hardware and software design, and allows for easy workflow modifications to meet future needs.

Samples are typically stored under at least four different conditions and analytical timepoints typically range from time 0 to 16 weeks. Analytics on all samples include Agilent 1200 configured for SEC- and IEC-HPLC, a Molecular Dynamics SpectraMax® UV-Vis Fluorescence microplate reader, Agilent's chip-based gel electrophoresis Bioanalyzer®, turbidity and visible particle analysis via digital image acquisition, pH, osmolality, and Wyatt DLS.

An example of the fully automated workflow can be shown with a formulation of antibody A. The stability of antibody A formulations was tested and the library design is shown in Figure 17. In an accelerated stress-testing study, these formulations were stored at -70 , 4 , 25 , and 37°C to show differences in stability. Samples were analyzed by pH, osmolality, electrophoresis (reduced and nonreduced), concentration (UV @ 280 nm), DLS, SEC, ion-exchange

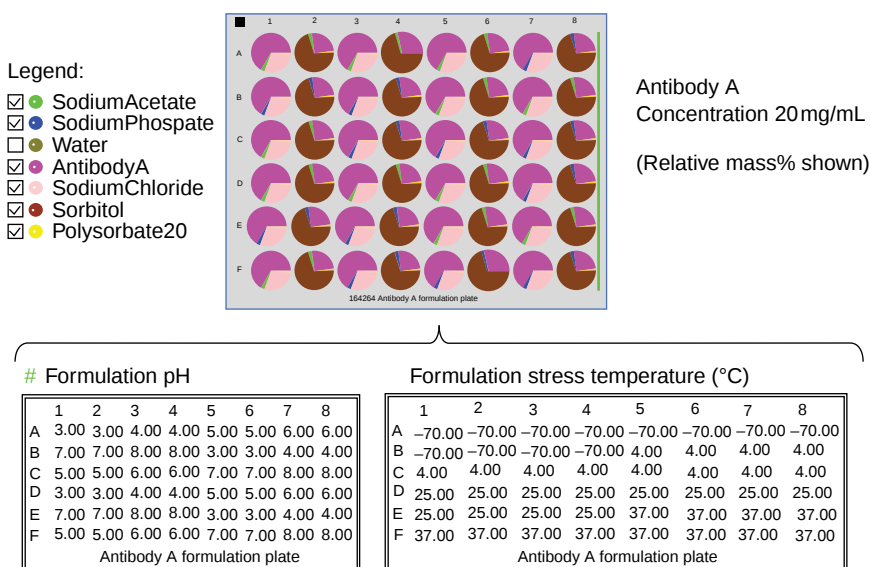


Figure 17 Graphical representation of library design of antibody A formulations. Weight percent of components shown with the aqueous buffer solutions suppressed.

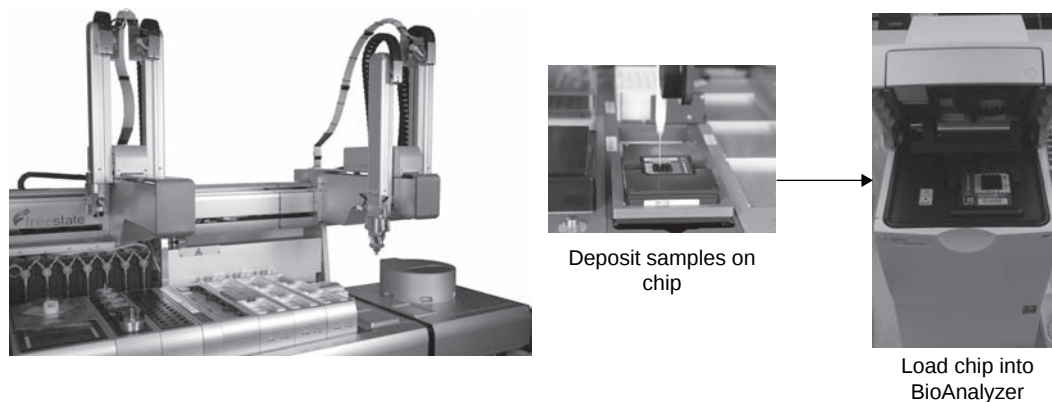


Figure 18 Freeslate Core Module for Bioanalyzer preparation equipped with a capped/decapper, 10 µL positive displacement tips, an automated centrifuge, a chilled receptacle, vortexing units, and a chip-priming syringe.

chromatography, and reverse-phase chromatography, with the system providing a fully automated work-up for each of these techniques, automatically passing samples to each analytical device, and automating the data collection from each station.

The value of full automation is shown when considering the integration of Agilent's chip-based gel electrophoresis Bioanalyzer®. The sample preparation for this station is complex in that it involves both work-up of the sample as well as preparation of the microfluidic chip. Automation becomes important when analyzing lots of samples because once primed with the gel, these chips need to be used within a certain window of time. Thus, a high throughput process needs to be able to automate the preparation of these chips. The workflow is shown graphically in Figure 19. In this case, the sample preparation is handled by an appropriately configured

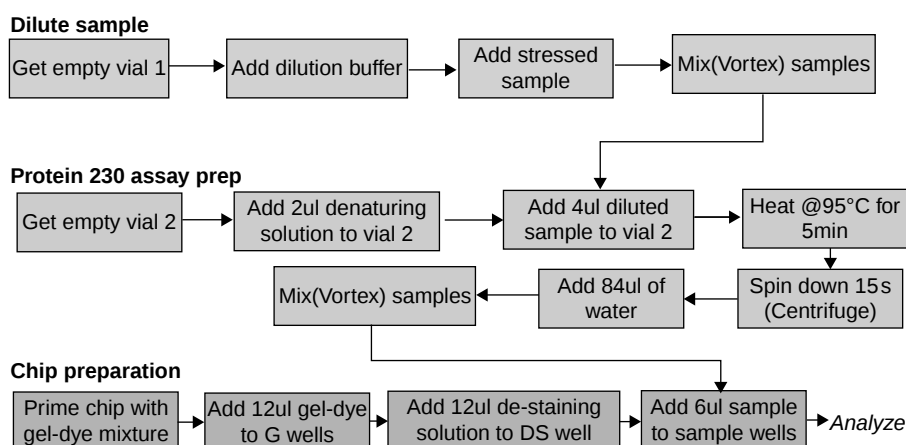


Figure 19 Block diagram of the automated workflow performed by the Core Module to prepare samples and chip for the Agilent Bioanalyzer.

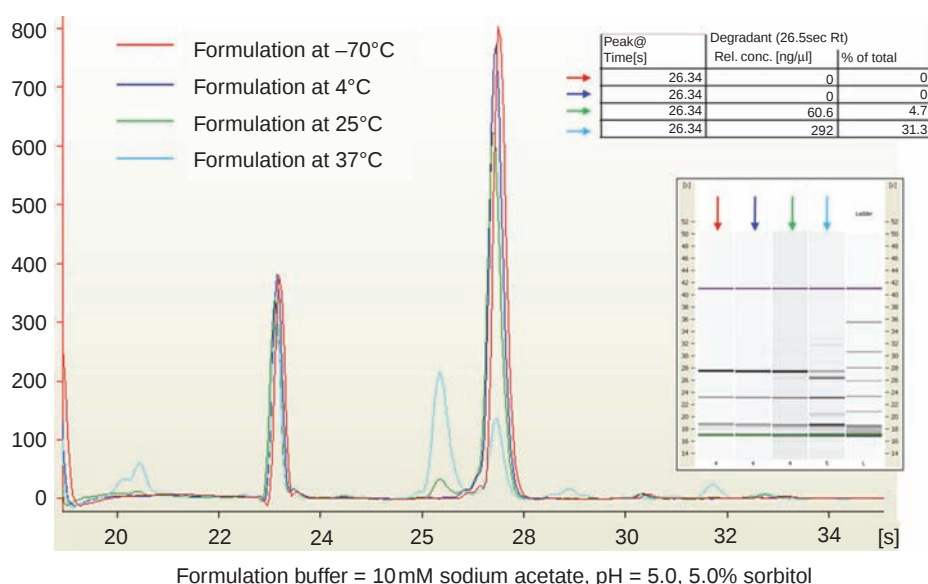


Figure 20 Results from Bioanalyzer® captured by LEA software. Informatics captures gel images, electropherograms, and quantitative results for both reduced and nonreduced conditions (only nonreduced shown).

Freeslate Core Module (Fig. 18). Data from this station have proven to provide consistent high quality allowing for quantitative analysis to be used. The stability of antibody A formulations as a function of temperature was studied, and the resulting data are shown in Figure 20.

This automated system, while representing a complex installation and significant capital investment, is able to increase the productivity of researchers by a factor 5×, allowing two researchers to fully explore and characterize about 2400 formulations per year. In addition, the system can accommodate different sample sizes and has the ability to handle microformulations as low as 125 μL per formulation, allowing one to pursue formulation development at an earlier stage of the development process.

GENERAL CONCLUSIONS

The pressure to produce more results with the same or with fewer resources will continue to encourage researchers to look for tools to improve efficiency and to extend productivity. The use of automation as a part of stress testing can be an effective tool to improve efficiency, to broaden the space explored, and to capture and standardize best practices. Automation will continue to benefit from technology improvements that will afford the ability to miniaturize experiments allowing one to explore a broader experimental space at an earlier stage of development. In addition, R&D labs are moving toward a paperless environment in which it will be easier work within distributed development teams in which scientists working manually seamlessly access data from fully automated systems in order to make development decisions and create reports.

ACKNOWLEDGMENTS

The authors would like to thank the following individuals for their contributions. Chris Wood from Pfizer, Bradley Campbell and Jerry Draper from Eli Lilly and Co, and Dr. Nallakan Arvindan and Jess Sager from Symyx Technologies, Dr. Byeong Chang from Integrity BioSolutions, and Stephen Cypes and Dr. Stephen Lambert from Freeslate, Inc.

REFERENCES

1. Di L, Kerns EH. Profiling drug-like properties in discovery research. *Curr Opin Chem Biol* 2003; 7: 402–8.
2. Fermier AM, Oyler AR, Armstrong BL, et al. Automation of chemical reaction kinetics and product distribution studies in pharmaceutical development. *J Assoc Lab Autom* 2002; 7: 83–8.
3. Sims JL, Robert JK, Bateman AG, et al. An automated workstation for forced degradation of active pharmaceutical ingredients. *J Pharm Sci* 2002; 91: 884–92.
4. Carlson E, Chandler W, Galdo I, et al. Automated integrated forced degradation and drug-exciipient compatibility studies. *J Assoc Lab Autom* 2005; 10: 374–80.
5. Strategic Directions International; Global Assessment Report v 10.5: Lab Automation & Life Science Instrumentation Industry, August 2009, 357–66.
6. Lina L, Rhodes T, Helmy R, et al. Poster at Eastern Analytical Society, Forced Degradation Automation. Sommerset, NJ, Nov 15–18, 2008.
7. SBS-format is a common lab automation format specifying the number of wells, the spacing, and substrate dimensions defined by the Society for Biomolecular Sciences.
8. Snyder LR, Kirkland JJ, Glajch JL. *Practical HPLC Method Development*, 2nd edn. New York: Wiley, 1997: 403–37.
9. Swartz M, Krull I. A Quality-by-design methodology for rapid LC method development: Part III. *LCGC N Am* 2009; 27: 328–39.
10. Al-Sayah MA, Rizos P, Antonucci V, et al. High throughput screening of active pharmaceutical ingredients by UPLC. *J Sep Sci* 2008; 31: 2167–72.
11. Xiao KP, Xiong Y, Liu FZ, et al. Efficient method development strategy for challenging separation of pharmaceutical molecules using advanced chromatographic technologies. *J Chromatogr A* 2007; 1163(1–2): 145–56.
12. <http://americanpharmaceuticalreview.com/ViewArticle.aspx?ContentID=541> (accessed December 2009).
13. Ruan J, Tattersall P, Losano R, et al. The role of forced degradation studies in stability indicating HPLC method development. *Am Pharm Rev* 2006; 9: 46–53.
14. Nelson ED, Harmon PA, et al. Evaluation of solution oxygenation requirements for azonitrile-based oxidative forced degradation studies of pharmaceutical compounds. *J Pharm Sci* 2006; 95: 1527–39.
15. Lilly Automation Takes the Stress Out of Stress Testing. *Molecular Connections* Fall 2008; 26: 10–15.
16. Roe SD. *Protein Purification Techniques*. New York: Oxford University Press, 2001: 4–10.

INTRODUCTION

Isothermal microcalorimetry (IMC) offers an attractive approach to stress testing because its measured parameter (heat change) is a near universal accompaniment to chemical or physical change. It is also nondiscriminating in terms of sample physical form (meaning that solids, liquids, or semi-solid materials can be analyzed directly) and the local environment in the sample ampoule is easily controlled (so it is easy to match classical stress conditions). Calorimetry is, therefore, ubiquitous and usable, in principle, from system to system and avoids the significant drawback of having to design assays specific to individual molecules. Furthermore, it can be a single, common thread running through compound development, in systems of increasing complexity.

Careful experimental design is a prerequisite in the interpretation of what could otherwise be complex data; in addition, recent advances in data analysis and interpretation methodologies can result in a description of the reaction process in terms of both thermodynamics and kinetics, the only technique for which such a complete, and direct, analysis is possible. It is the purpose of this chapter to discuss the principles of the technique and, through discussion of literature examples, examine its role in stress testing of pharmaceuticals.

Heat as an Indicator of Change

Changes in enthalpy (q , in Joules, J) are the universal accompaniment to reaction (there are very few thermo-neutral reactions), making determinations of enthalpy changes attractive in analytical and physical chemical applications and it is a suitable signal to highlight any changes that may occur in a sample. Since, necessarily, calorimetric measurements must be made as a function of time, calorimeters record an output of *power* (dq/dt , given the SI unit of Watts, W; note that 1 W is equivalent to 1 J s^{-1}). Thus, the raw output from a calorimetric experiment is a plot of power versus time (often called a thermogram). Integration of the data results in the total quantity of heat released (Q).

The universal nature of heat [as eloquently phrased, heat does not come in different colors (1)] is also the technique's biggest drawback. It is often the case that calorimetric data are complex in form and derive from several simultaneous (including both physical and chemical) processes. Furthermore, calorimetric data are extremely (and perhaps uniquely) susceptible to systematic errors introduced by the accidental measurement of one or more of a range of processes (such as solvent evaporation and/or condensation, erosion, side reactions, and so on) that may occur concurrently with the study process(es). Care must therefore be taken to ensure that erroneous or unexpected powers have not been accidentally introduced as a corollary of poor experimental design or execution. This is the main reason why calorimetric data need to be analyzed assiduously, often in combination with other complementary data.

Principles of Microcalorimetry

There are only three methods by which heat change can be measured, and any commercial instrument will be based on one of the following principles.

Power Compensation Calorimeters

In power compensation calorimetry, an electrical element is used either to add or remove heat from the calorimetric vessel as the sample reacts, maintaining the sample and vessel at a given

temperature. The power output from the sample is thus the inverse of the power supplied by the element. In order to be able to heat and cool, the element is usually based on the Peltier principle. A typical application of this type of calorimetry is power compensation differential scanning calorimetry (DSC).

Adiabatic Calorimetry

In an ideal adiabatic calorimeter, there is no heat exchange between the calorimetric vessel and its surroundings. This is usually attained by placing an adiabatic shield around the vessel. Thus, any change in the heat content of a sample as it reacts causes either a temperature rise (exothermic processes) or fall (endothermic processes). The change in heat is then equal to the product of the temperature change and an experimentally determined proportionality constant (or calibration constant, ϵ , which is the effective heat capacity of the system). The proportionality constant is usually determined by electrical calibration.

In practice, true adiabatic conditions are difficult to achieve and there is usually some heat leak to the surroundings. If this heat leak is designed into the calorimeter, the system operates under semi-adiabatic (or isoperibol) conditions and corrections must be made in order to return accurate data. These corrections are usually based on Newton's law of cooling [the most common being the method of Regnault-Pfaundler (2)]. This type of system is commonly used for solution calorimetry.

Heat Conduction Calorimeters

A heat conduction calorimeter surrounds the sample with a heat sink, which acts to maintain the system at a constant temperature. Between the vessel and the heat sink is a thermopile wall. Any heat released or absorbed upon reaction is quantitatively exchanged with the heat sink. The thermopiles generate a voltage signal that is proportional to the power flowing across them; this signal is amplified, multiplied by the cell constant (determined through electrical calibration), and recorded as power versus time. An isothermal system is not limited to reaction processes that reach completion within a short time, as semi-adiabatic instruments are, because it is always (essentially) in equilibrium with its surrounding heat sink. Furthermore, the greater measuring sensitivity of the thermopiles (as opposed to the thermistors used in semi-adiabatic instruments) means that smaller sample masses can be used.

A modern heat conduction, isothermal microcalorimeter (such as a Thermal Activity Monitor, TAM, TA Instruments LLC) is capable of maintaining a baseline of ± 0.1 mW with a temperature stability of $\pm 0.0001^\circ\text{C}$. A schematic representation of the TAM is given in Figure 1 (courtesy of TA Instruments LLC). The instrument consists of a large thermostatted water bath into which are placed four or fewer calorimetric channels. Each channel comprises two chambers: a reference chamber and a sample chamber, Figure 2 (courtesy of TA Instruments LLC). Each chamber can accept glass vials (3 mL capacity), stainless steel ampoules (5 mL capacity) or, indeed, any ampoule designed to permit the study of specific reaction systems.

Isothermal microcalorimeters are usually operated at, or around, ambient temperatures and employ their inherent sensitivity to detect the heat-flow signals from any processes that are occurring in the sample ampoule. A comparison of the performance of a standard DSC (a Perkin-Elmer DSC 7, for instance) with that of a TAM gives an idea of the sensitivity of an isothermal microcalorimeter. Table 1 shows the typical performance parameters for these two instruments and shows that the TAM is of the order of 10,000-fold more sensitive than a standard DSC (3). DSC has been employed for accelerated stress testing because the rates of the processes under observation are accelerated at higher temperatures. The data that relate to any specified storage conditions are determined by extrapolation via application of the Arrhenius equation. This enforces the assumption that any processes that occur at the storage temperature are the same as that which occur over the experimental temperature range. Of course, this is not necessarily the case. Examples of processes which may lead to such problems include phase changes, moisture transfer (e.g., excipient drug), etc.

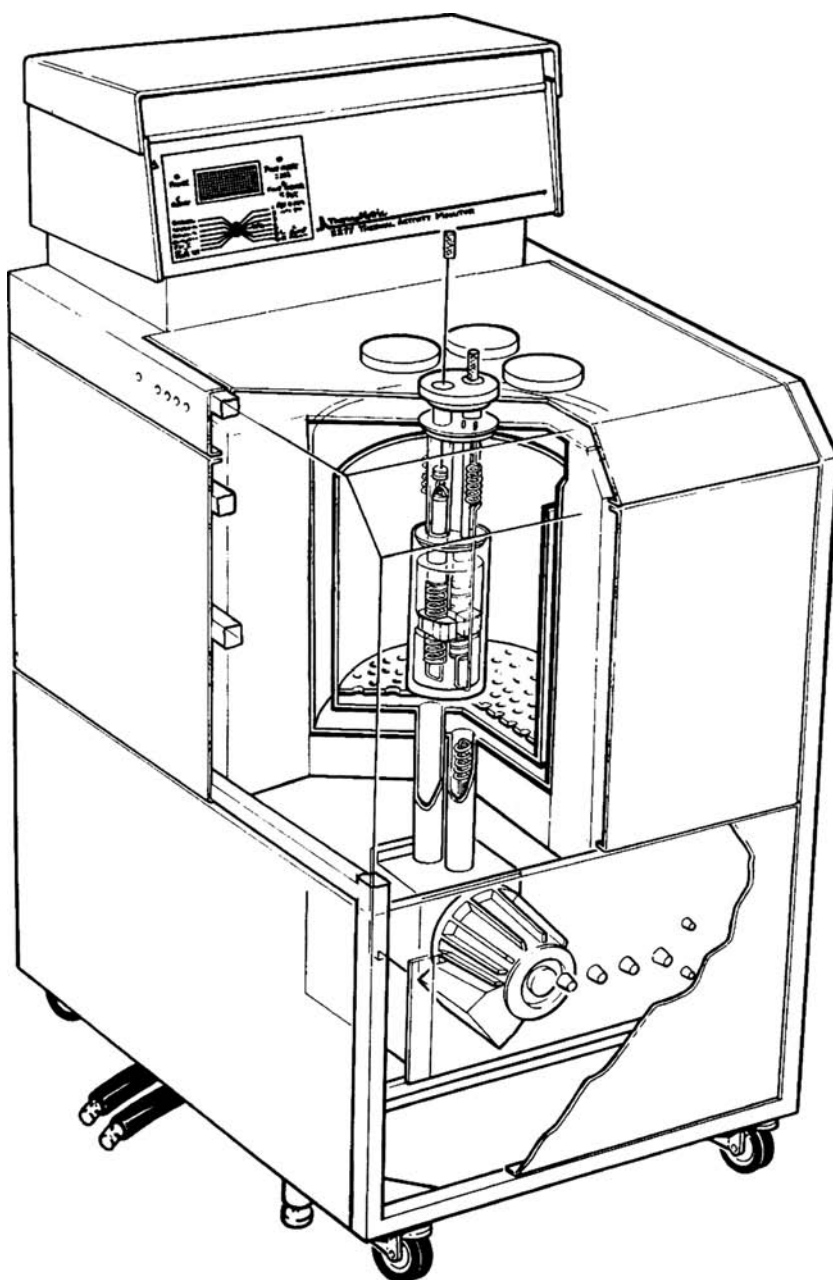


Figure 1 A schematic representation of a TAM (courtesy of TA instruments LLC).

The data in Table 2 show the temperature rise that would be required to accelerate first-order reactions with activation enthalpies of 50, 75, and 100 kJ mol⁻¹ by factors of between 1 and 100,000 (4). The data show, for example, that if a reaction of rate 1 (arbitrary units) and activation enthalpy of 50 kJ mol⁻¹ is observable in a TAM at 20°C, then for this reaction to be observed in a DSC the temperature will have to be raised by 239°C. Such an extrapolation should not be undertaken without caution.

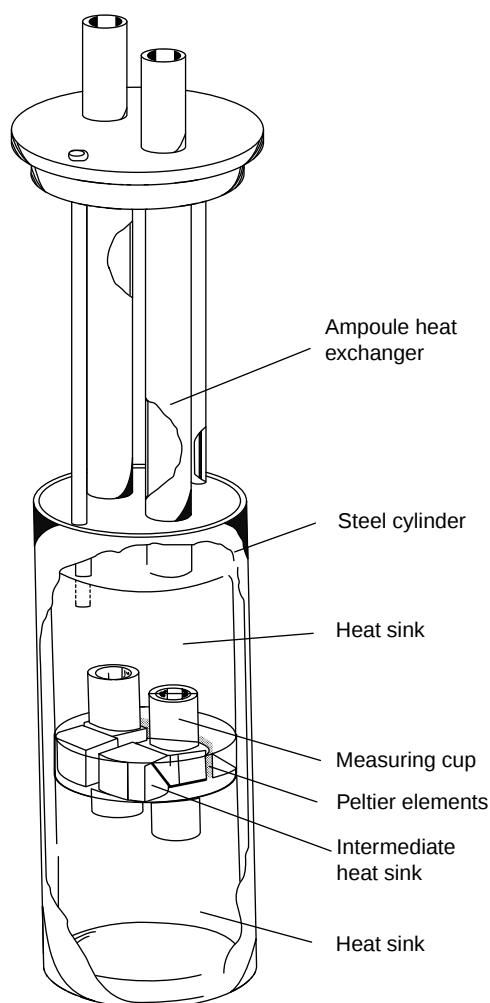


Figure 2 A schematic representation of a TAM channel (courtesy of TA instruments LLC).

Table 1 Comparison of Differential Calorimetry and Isothermal Microcalorimetry (3)

	DSC	TAM
Sensitivity (mW)	10	0.1
Sample mass (mg)	10	1000
Specific sensitivity (W/g)	1	0.0001

Calorimeter Selection for Pharmaceutical Studies

There is clearly a wide range of calorimetric instrumentation available and it is important to select the most appropriate type for a particular sample. The ubiquitous nature of heat means that calorimetric measurements are more prone to systematic and operator-induced error than most other analytical techniques; these errors become proportionately more significant as the

Table 2 The Temperature Increase, Over 20°C, that Would Be Required to Accelerate a Reaction by the Specified Factors (4)

Factor of Rate Increase	Activation Enthalpies (kJ mol ⁻¹)		
	50	75	100
1	0	0	0
10	37	24	17
100	85	52	37
1000	149	85	59
10000	239	125	85
100000	375	175	114

heat output from the study sample decreases. Moreover, the design of many instruments is a compromise between factors desirable for good calorimetric measurement and factors necessary to ensure a certain measurement function (5), meaning that careful experimental design and suitable calibration (preferentially using chemical test reactions) are essential precursors to accurate sample measurement.

Adiabatic (temperature change) calorimeters are limited to the study of events that progress to completion within 1–2 hours, because of the inherent difficulties in making the necessary heat-loss corrections over longer time periods (6). Practically, this means that adiabatic calorimeters are not suited to pharmaceutical stability assays, where degradation rates of 1–2% per year or less may reasonably be expected. However, adiabatic calorimeters have found widespread application in pharmaceutical preformulation because they aid physical characterization of APIs and excipients, and the number of applications of the technique is growing. Examples from the recent literature include its use to detect polymorphs (7), to rank the stability of polymorphs (8), to investigate interactions between species (9–11), to quantify small amorphous contents (12,13), and to study the formation of liposomes (14). In these types of experiments, reaction is initiated by breaking an ampoule containing (usually) a solid sample into a reservoir of liquid; they are therefore often referred to as solution calorimeters.

Both power compensation and heat conduction calorimeters are suited to the study of long-term processes. Power compensation instruments have a poorer detection limit but a better capacity to cope with high powers and are therefore usually used to study energetic reactions with large heat rates (15). The power compensation principle also underpins many DSC designs. Heat conduction calorimeters, because they can measure micro and even nanowatts are most suited, and most commonly used, for the study of long-term, low-energy reactions, typified by the degradation mechanisms often followed by pharmaceuticals.

Common Experimental Configurations

Batch or Ampoule

Ampoule calorimetry is the simplest and probably most commonly employed method for loading samples; sample and reference materials are placed into sealed ampoules before being loaded into the calorimeter. Ampoules can be reusable or disposable, can vary in volume, and can be constructed from a variety of materials (typically glass or metal). There are many advantages to using ampoules to load samples (as opposed to loading samples directly into a calorimetric chamber), the biggest being that it affords the opportunity to study virtually any sample because it is easily removed from the instrument after measurement. Pharmaceutically this means that it is possible directly to study heterogeneous systems, such as creams and emulsions, as well as solids and liquids; calorimetry is one of the few analytical tools that afford such utility. It is also possible to control the local environment (i.e., the relative humidity) in the

ampoule. Furthermore, it is possible to construct specific apparatus that will fit within the calorimeter for specific analyses (such as titration or photocalorimetry).

The main drawback of removable ampoules is that the thermal contact between the ampoule and the calorimeter will vary with each use, increasing noise in the raw data signal. There is also the possibility that the sample and reference ampoules will be mismatched, further reducing the quality of the experimental data, and that additional process (such as relaxation of rubber seals) will contribute a power to the measured signal.

In batch calorimetry, the reaction vessel is divided into two compartments that are connected by an air space. Both compartments can be charged with sample (either a solid or a liquid). Once thermal equilibrium has been attained, the contents of the two compartments are mixed, usually by rotating the vessel (hence they are also known as mixing calorimeters), and the heat of interaction is measured.

Flow Calorimetry

In flow calorimetry, as is implied by the name, a liquid flows through the calorimetric vessel. Solutions are held in a reservoir external to the instrument and a peristaltic pump is used to circulate the liquid. Two operational modes are available: flow through and flow mix. In flow-through operation a solution is pumped from an external reservoir, passes through the calorimetric vessel, and is returned either to the initial reservoir or to waste. In flow-mix operation, two (usually) liquids are pumped into the calorimetric vessel where they are mixed and then pumped to waste. Thus, in flow-through operation, the calorimeter measures the power output from a system after mixing and in flow-mix operation the calorimeter measures the power associated with the mixing process itself.

Gas Perfusion Calorimetry

In gas perfusion calorimetry (a schematic diagram of a commercial unit is given in Figure 3, courtesy of TA Instruments LLC), a gas is flowed over a sample; the gas can be dry or can carry water or solvent vapors (typically water or ethanol) and the RH or RVP (relative vapor pressure) can be controlled. Typical examples of the use of perfusion calorimetry include using the vapor to probe specific binding sites on the surface of the sample (for instance, acidic or basic sites), wetting the sample to induce the formation of hydrates or solvates, and increasing the partial pressure of a vapor to induce recrystallization of an amorphous material. The RVP of the probe gas can be altered, either in discrete steps or in a linear ramp. This is usually achieved by using mass flow controllers to mix quantitatively two vapor streams flowing from different sources: one dry and one saturated with vapor.

If it is only necessary to maintain the sample under only one, specific, partial pressure, then a much simpler methodology may be employed. This involves the placement of a small glass tube (variously known as a Durham tube, a hydrostat or a hygostat) holding a small quantity of solvent within an air-tight ampoule containing the sample. Saturated salt solutions will maintain a constant RH within a confined space at equilibrium. The specific RH attained is dependent on the ambient temperature and salt used (16,17). Some typical salts and the RH values they maintain are given in Table 3.

Solution Calorimetry

Solution calorimetry is taken here to mean the measurement of the heat change when a solute (usually a solid but liquids may also be used) is dispersed in a large volume of solvent (to ensure complete dissolution). Usually this is achieved using "ampoule breaking" instrumentation (wherein the solute is held in an ampoule which is mechanically broken into the solvent reservoir). The area under the dissolution peak is the enthalpy of solution of the solute in the solvent.

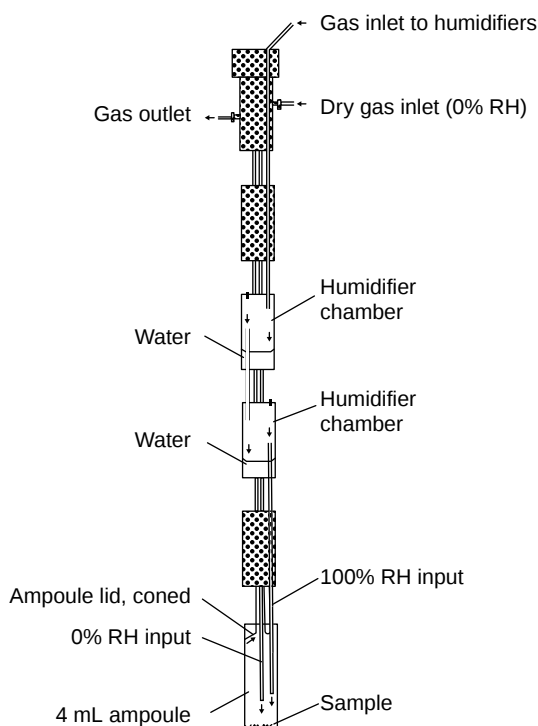


Figure 3 A schematic representation of a gas perfusion unit (courtesy of TA instruments LLC).

Table 3 The Relative Humidities (RH) Obtained at Various Temperatures Using Saturated Salt Solutions (17)

Salt	Temperature (°C)		
	25	35	45
LiCl	11.3	11.2	11.2
MgCl ₂	32.8	32.0	31.1
NaBr	57.5	54.0	52.0
NaCl	75.3	74.8	74.7
KCl	84.3	82.9	81.7

Isothermal Titration Calorimetry

In isothermal titration calorimetry (ITC), small aliquots of a titrant solution (held in a reservoir external to the instrument) are added in sequential aliquots to a solution held within the calorimetric vessel and the heat change per injection is recorded. In a typical experiment, up to 30 injections (~10–15 μL each) are made into the liquid reservoir. Usually, titration calorimetry is used to study the binding interaction between a ligand (a drug or potential drug candidate) and a substrate (typically a protein, enzyme, or some other biological target), although of course the technique is not limited to this area.

In a typical binding experiment, the area under each peak gives the heat change per injection of titrant and can be plotted against the number of moles of titrant injected (or concentration of titrant in the vessel) to yield a binding isotherm. It is important to recognize that if the

interaction under investigation involves binding, then, in order to analyze the data properly, it must be ensured that the number of moles of ligand injected is sufficient to ensure that all the binding sites on the target are occupied; this is shown by later injections reaching a minimum, and consistent, value.

It is also important to note that the measured heats from such an experiment comprise contributions from many effects, such as dilution (both of ligand and receptor) and mixing, and care must be taken to ensure that the proper reference experiments have been conducted (for accurate work three are required; solvent into solvent, ligand into solvent, and solvent into receptor). Assuming that both the ligand and receptor are prepared in the same solution (usually a buffer; note that the mixing of samples prepared in different buffers can often cause a large heat effect that arises from the ionization of the buffer species), then the greatest additional contribution to the measured heat is the enthalpy of dilution of the ligand solution. This results from the need to prepare relatively concentrated solutions for injection because the volumes injected are so small.

If properly constructed, the principal parameter returned from a binding experiment is then the binding constant (K_b). This is usually obtained by nonlinear analysis of the binding isotherm.

APPLICATION TO STRESS TESTING

The primary focus of any stress test is to ascertain whether a compound, either alone or formulated with other actives and/or excipients, will degrade significantly over a defined period of time under specified environmental conditions. If no degradation is observed, then the compound is assumed to be stable and no further assessment is necessary. If degradation is observed then either the system is abandoned or further experimentation is required so as to identify the cause (which may not necessarily arise from a chemical change in the sample; physical changes are likely in amorphous or polymorphic drugs or in heterogeneous drug delivery systems). In this sense, tests can therefore be qualitative (i.e., designed to give a yes/no, stable/unstable answer) or quantitative (i.e., designed to return a rate constant); careful experimental design allows IMC to conduct both types of assessment.

Primary Screening

A primary screen is taken here to mean a quick assessment of the stability of a compound or mixture during preformulation or early formulation; the output is generally qualitative, the formulator simply requiring a stable/unstable answer. Degradation of an API or excipient will almost certainly lead to a loss of potency; since the lowest acceptable level of potency is usually 90% of the label claim (18), IMC must be able to detect reactions on this order to be of practical value. It has been shown, by analysis of 50 hour of power-time data recorded at 25°C using an isothermal calorimeter for a reaction following first-order kinetics and occurring with a reasonable ΔH of -50 kJ mol^{-1} , that it is possible to distinguish between rate constants of 1×10^{-11} and $2 \times 10^{-11} \text{ s}^{-1}$ (19). A reaction progressing with a rate constant of $1 \times 10^{-11} \text{ s}^{-1}$ has a half-life of approximately 2200 years.

Thus, an initial screen of a sample should detect rapidly any degradation processes occurring, and the absence of any heat changes gives confidence that the system under investigation is stable (although it should be noted that exothermic and endothermic powers occurring simultaneously will reduce the observed net power signal).

The experimental protocol for the case of a single component is very simple and is represented by the flow diagram in Figure 4. Such an approach was first described by Pikal (20), who showed there was a correlation between the exothermic heat output of some pharmaceutical systems and their known degradation rates (previously determined using other analytical methods). The data also showed that degradation rates of the order of 2% per year were easily quantified.

A similar approach can be adopted for testing binary mixtures of an API with an excipient; a typical process is represented by the flow diagram in Figure 5. In this case,

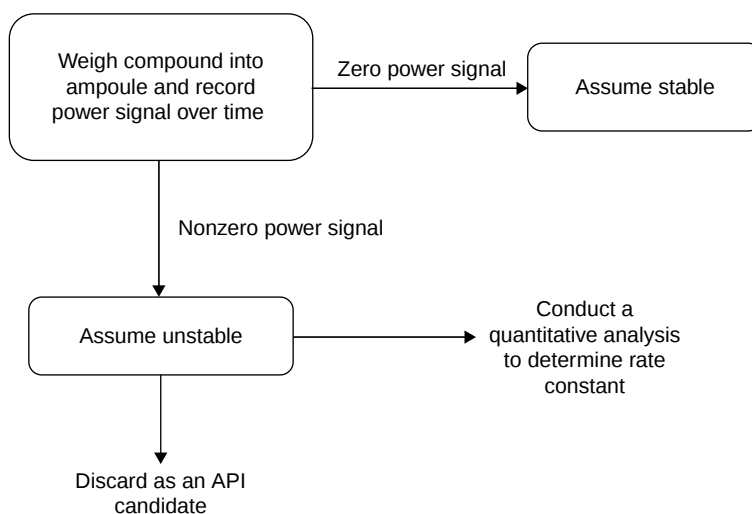


Figure 4 A flow diagram for a qualitative stability screen for an API alone.

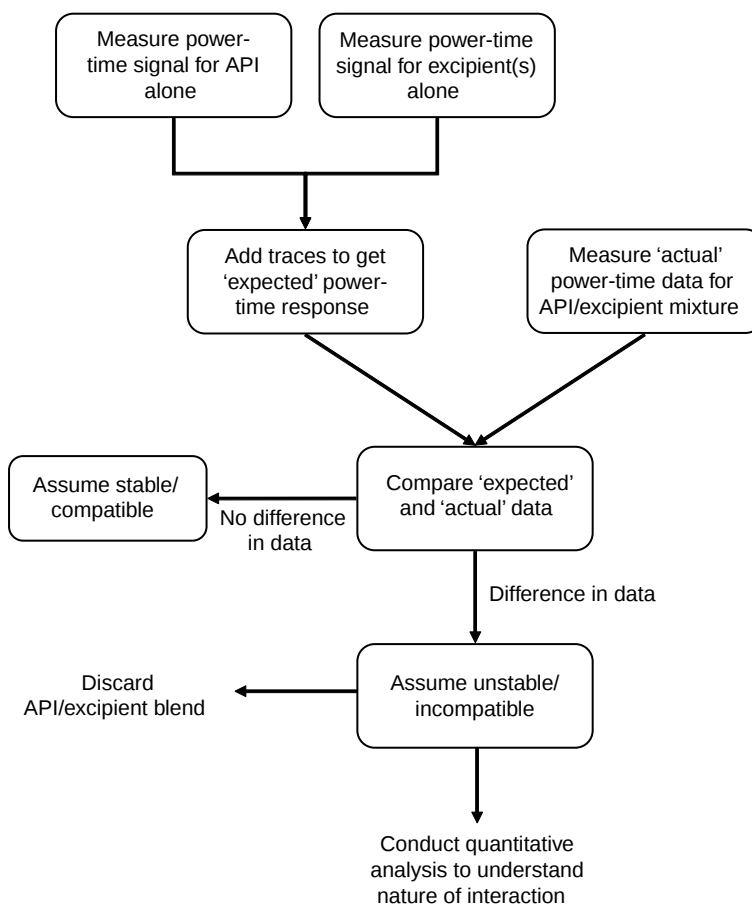


Figure 5 A flow diagram for a qualitative stability screen for a binary mixture of API and excipient.

however, a number of control experiments must be performed first. The thermal response of the active alone and the excipient alone are recorded and then summed, to give the “predicted” power response. This trace is then compared with that determined for the actual drug–excipient mixture. Any unexpected powers recorded in the drug–excipient mixture indicate a possible interaction.

This approach has been discussed in the literature (21,22) and provides an extremely versatile screening methodology. However, small variations between the experimental and the predicted data sets are often observed in these experiments. These can arise from various experimental factors, such as small differences in sample masses or variations in the position of the baseline caused by different materials. One approach, therefore, is to set arbitrary upper and lower limits (which can be a power value, such as $\pm 10 \mu\text{W}$, or a percentage value, such as $\pm 10\%$); if the predicted and experimental data differ by more than the limits the mixture is discarded.

Calorimetric measurements have been used to screen batch-to-batch variations in excipients (23). In this work, the authors used solution calorimetry to measure the heat of solution of a number of excipients (cetostearyl alcohol, microcrystalline cellulose, and a number of nonionic surfactants) and showed that batch variations could be quantified.

A further adaptation of this approach is to use water slurries instead of humidified samples (24). For instance, Schmitt et al. (25) developed a procedure that allows rapid assessment of API–excipient compatibility by studying two developmental drugs formulated with excipients that could undergo a Maillard reaction. Their recommended methodology is to add water (20% w/w) to a binary mixture of API and excipient (100 mg of each) and monitor the power-time signal at 50°C for 3 days. They note that comparison of the calorimetric results with actual formulation stability showed that it was possible to predict relative stability within functional classes, but advised caution because the apparent reaction enthalpies varied threefold among excipients within the same functional class.

Note here that primary screens, such as those described above, are usually designed to *maximize* the chance of seeing an interaction if there is one, rather than simulate the conditions to be found in the intended formulation; thus, binary samples are usually mixed in a 1:1 by mass ratio, ensuring that the two materials have equivalent particle sizes, and a high RH may be used to facilitate reaction. Further screens under more representative conditions can then be conducted if necessary to see whether any instability observed in the primary screen is relevant to the intended formulation.

The approach adopted in the studies discussed above does not allow quantification of the amount of degradation (unless complete degradation occurs within the time frame of the experiment, in which case the use of fractional areas allows the calculation of percent degradation with time) nor, without further analysis, does it indicate the exact nature of any degradation processes. It does, however, allow an initial judgment to be made on the likely stability of a compound or mixture, giving the formulator a valuable insight into the stability of the API to be formulated or which excipients are likely to result in a stable product.

The lack of quantitative studies is a direct result of the difficulties that arise in the analysis of complex power-time signals: for instance, a low enthalpy, fast rate process may easily appear the same as a high-enthalpy, slow-rate process (26), or competing endothermic and exothermic processes may result in an apparently small thermal response (27). These problems were noted in the study of API–excipient stability using water slurries by Schmitt et al. (25), and made direct stability assessment comparisons between samples difficult. While a slow rate process may have no consequence on product stability, a fast rate process may be of some considerable significance and it is clear that an analysis that results in the derivation of a rate constant, reaction enthalpy, or both is necessary for true comparisons to be drawn. Indeed, it has been stated that although IMC offers considerable benefits in determining product stability, ultimately it will never replace the need for chemical analysis (28); this area forms a considerable challenge that must be overcome if IMC is to become more widely used for pharmaceutical stability assessment.

Stress Testing of Individual Compounds

If the primary screen conducted in the calorimeter indicates some instability in the sample, and there is a need to understand the process on a molecular level or define its reaction kinetics and/or thermodynamics, then the traditional approach is to conduct further analyses with complementary analytical techniques. However, it is usually the case that the power-time data so obtained already contain sufficient information that, if a suitable analysis can be undertaken, the process(es) occurring can be quantified with no further experimentation.

For instance, Koenigbauer et al. (29) determined the activation energies for the degradation of several drugs, including phenytoin, triamterene, digoxin, tetracycline, theophylline, and diltiazem, using the initial power rates, measured using IMC at several elevated temperatures. The results were compared with HPLC data recorded at a single temperature and it was shown that the IMC data were more precise. Similarly, Hansen et al. (30) showed that the shelf-life of a product, degrading via an autocatalytic reaction, was inversely proportional to the rate of heat production during the induction period, using lovastatin as an example.

If there is only one reacting component, then the reaction order will be integral and it is relatively easy to determine the rate constant by replotting the data.

Thus;

- If the power signal is constant as a function of time, then the process is zero-order. The rate constant can be determined from the power value if the number of moles of material and reaction enthalpy are known (the deflection is equal to $k \cdot \Delta H \cdot V$ (where V is the volume of sample))
- If a plot of $\ln(\text{power})$ versus time is linear, then the process is first order and the rate constant is given by the gradient of the line
- If a plot of $\text{power}^{-0.5}$ versus time is linear, then the process is second order and the rate constant is given by the gradient of the line

If the data do not appear to be zero-, first- or second-order, there may be multiple processes occurring or (unusually) the process may have a non-integral order.

The following text discusses applications to real data, based on the most common degradation pathways; hydrolysis, oxidation, elevated RH, and photodegradation.

Hydrolysis Reactions

Many pharmaceuticals are susceptible to hydrolysis and, since water is difficult to remove entirely from a formulation, hydrolysis is a common cause of chemical instability. There are numerous examples in the literature where hydrolysis reactions have been studied calorimetrically. For instance, the degradation rate of meclofenoxate hydrochloride (MF), which hydrolyses in aqueous solution, has been determined with IMC (31). By plotting $\ln(\text{power})$ versus time, the degradation rate constants for MF at pH 6.4 and 2.9 were determined to equal $1.14 \times 10^{-4} \text{ s}^{-1}$ and $9.7 \times 10^{-7} \text{ s}^{-1}$, respectively. Comparison of these data with rate constant values determined using HPLC ($1.29 \times 10^{-4} \text{ s}^{-1}$ and $9.0 \times 10^{-7} \text{ s}^{-1}$) revealed the utility of the calorimetric technique. A similar approach has been used to determine the rate constants for ampicillin degradation in aqueous buffers (32) from pH 2 to 8, a number of cephalosporins (33) and diclofenac sodium (34).

Simoncic et al. (35) used IMC to measure the hydrolysis of perindopril erbumine at pH 6.8; kinetic analysis of the data revealed only one reaction pathway (hydrolysis) suggesting that the alternative degradation route (cyclization) was not occurring.

Kinetic models have also been applied to systems degrading via hydrolyses. Angberg and Nyström (36) studied aspirin hydrolysis in aqueous solution as a function of pH. Rate constants were derived from the gradient of $\ln(\text{power})$ versus time plots at a series of temperatures (30–50°C). In 0.1 M HCl at 40°C, the rate constant was $9.0 \times 10^{-6} \text{ s}^{-1}$, increasing to $22.5 \times 10^{-6} \text{ s}^{-1}$ at 50°C, while in pH 4.8 acetic acid buffer rate constants of $14 \times 10^{-6} \text{ s}^{-1}$ and $34.1 \times 10^{-6} \text{ s}^{-1}$ were

determined at 40 and 50°C, respectively (37). Using the Arrhenius relationship, a rate constant for aspirin degradation at 25°C in 0.1 M HCl of $2.3 \times 10^{-6} \text{ s}^{-1}$ was predicted. Skaria and Gaisford (38) were able to record data for aspirin hydrolyzing directly at 25°C, recording a rate constant of $2.8 \times 10^{-6} \text{ s}^{-1}$.

A typical formulated pharmaceutical may well have several independently degrading components and, although the degradation kinetics of the individual components of a medicine may be known, their behavior in combination may be significantly different. It is therefore not sufficient simply to know the stability profiles of the individual materials but to understand any synergism between them. The overall degradation profile for a mixture of compounds can be described by summation of the relevant kinetic mechanisms. In terms of a calorimetric analysis, this means data can be modeled by summation of the relevant kinetic expressions.

An example of this approach to analyze compounds degrading in parallel has been reported by Skaria et al. (39) who studied aqueous solutions of binary mixtures of selected parabens. Their methodology allowed determination of the first-order rate constant and reaction enthalpy for degradation of each component, so long as one rate constant was at least twice the magnitude of the other. It was found that the reactions did not need to run to completion in order for the analysis to be successful; a minimum of 15 min of data were required for samples with one degrading component and a minimum of 4 hours of data were required for samples with two degrading components. It was observed that the rate constants for paraben degradation in binary systems were significantly lower than expected. This was ascribed to the fact that the parabens degrade to a common product and is an important factor that should be accounted for when the two or more parabens are formulated together.

Oxidation

Since most calorimeters employ closed ampoules, it is possible to control the local atmosphere in the sample cell and hence to study oxidation reactions. Two simple methodologies to determine whether a sample is decomposing via oxidative degradation are as follows:

- To run samples in closed ampoules under either air or nitrogen, differing power-time profiles indicates that oxidation is occurring. Alternatively, air or dry nitrogen gas can be flowed over the sample with a gas perfusion unit.
- For drugs in solution, to add a metal chelating agent (such as EDTA) which will bind any free metal ions that may catalyze oxidation; differing power-time profiles in the presence and absence of chelating agent indicates that metal-catalyzed oxidation is occurring.

For instance, Otsuka et al. (31) investigated the oxidation of *dl*- α -tocopherol using IMC. Samples of the drug (a slightly viscous liquid) were placed in glass ampoules that were left open to the atmosphere in ovens at 50, 40, 30, and 23°C for varying lengths of time. Each sample was then capped before being placed in the calorimeter. Equivalent samples were analyzed using HPLC. First-order rate constants for the samples were determined at each temperature. An Arrhenius plot of the data revealed a linear correlation and an excellent agreement between the HPLC and calorimetric data.

Another example of the use of calorimetry in this way to study oxidative degradation is provided by Tan et al. (40) who studied tretinoin (all-trans-retinoic acid). Tretinoin degrades upon exposure to heat, light, and/or oxygen principally to form one of two geometrical isomers, 13-*cis*-retinoic acid (isotretinoin), or 9-*cis*-retinoic acid. While isotretinoin exhibits almost the same clinical activity as tretinoin, and indeed is formulated for systemic administration, 9-*cis*-retinoic acid is clinically inactive and, hence, the precise quantification of the rates of formation of the breakdown products is of considerable importance. It was demonstrated that under air the decomposition of isotretinoin was autocatalytic while the decomposition of tretinoin followed zero-order kinetics. In both cases, HPLC analysis showed the appearance of degradation products, although the mechanisms were complex. Under a nitrogen atmosphere

both compounds showed a first-order kinetic event, but simultaneous HPLC analysis showed no evidence of chemical degradation, indicating that a physical change was occurring.

A similar approach has shown that solid-state lovastatin degrades in the presence of oxygen (41). In this case, the degradation mechanisms were shown to change between 50 and 60°C, an important observation in the context of elevated temperature studies, as discussed earlier.

Elevated RH Studies

Proper control of the water content of a formulation, or control over the supply of water to a formulation during storage, is vital if stability is to be assured. This is because water acts in many ways to degrade pharmaceuticals (which include both chemical and physical mechanisms). Water may, for instance, induce degradation via hydrolysis, cause an amorphous sample to recrystallize by lowering its glass transition (T_g) temperature, cause deliquescence of crystals, result in the collapse of a freeze-dried "cake," result in the formation of hydrates or act as an intermediary between two solid components. It is therefore essential to know how a sample will behave in the presence of water and, if necessary, reformulate or repackage the product to ensure that there is no loss of potency upon storage.

The use of IMC to investigate the stability of pharmaceuticals in the presence of water is possible because experiments can be constructed where the RH in the sample ampoule can be accurately controlled. Thus, an IMC approach allows measurement of stability under such conditions *directly* (for most other analytical techniques, the sample must be stressed under an elevated RH prior to measurement). As should also be familiar by now, calorimetric data also permit the quantification of both chemical and physical change, a facility perhaps most important to stability assessment under humid conditions.

Gas perfusion calorimetry has been used to measure the interactions between water vapor and a number of amorphous pharmaceutical solids (sucrose, lactose, raffinose, and sodium indomethacin) (42). The power-time data exhibited general trends that aided an explanation of the effect of moisture content on the physical stability on the amorphous form at given storage temperatures. At some RH threshold (RH_m), the data showed a large increase in the energy of interaction between the water vapor and the sample that could not be explained by a phase or morphology change. Below RH_m water sorption/desorption was reversible; above RH_m hysteresis was noted and water–water interactions dominated the thermal response. Samples stored in an atmosphere below RH_m showed no evidence of instability after several months.

Polymorph Stability

Polymorph screening will have been conducted early in development and it may well be the case that a metastable form has been selected for development. This being so, understanding the rate of conversion to a more stable form, especially under stress from temperature or RH, is critical in ensuring regulatory approval of the product.

Isothermal calorimetry has found application to stress testing of polymorphic forms, if the change in form occurs over a reasonable time period (days to weeks under stress conditions). This is because in order to affect quantitative analysis the total heat released upon form change is required. An example is provided by Urakami and Beezer (43), who studied form change in seratrovastatin, an anti-asthmatic drug. The conversion of form II to form I, studied at 58°C and 63% RH, was seen to produce an exothermic peak over ~1 day. The speed of the conversion was closely linked to the particle size of the drug, greater surface areas resulting in faster conversion rates (Fig. 6). The kinetics of solid-state reactions are described in terms of a fraction reacted (α) versus time. This is easily calculated from the calorimetric data since it is the ratio of the heat released to time $t(q)$ to the total heat released (Q). Urakami and Beezer (43) performed this conversion and then fitted the data to a kinetic model to recover rate constants

and reaction enthalpies as a function of particle size, obtaining the data in Table 4. Repeating the measurements as a function of temperature and humidity gave a stability profile over storage conditions (Table 5).

Photostability

There are a number of reasons why an assessment of photostability may be desirable; to quantify the stability of a drug or product under “whole light” conditions, to determine stability as a function of wavelength (which may give mechanistic information on the

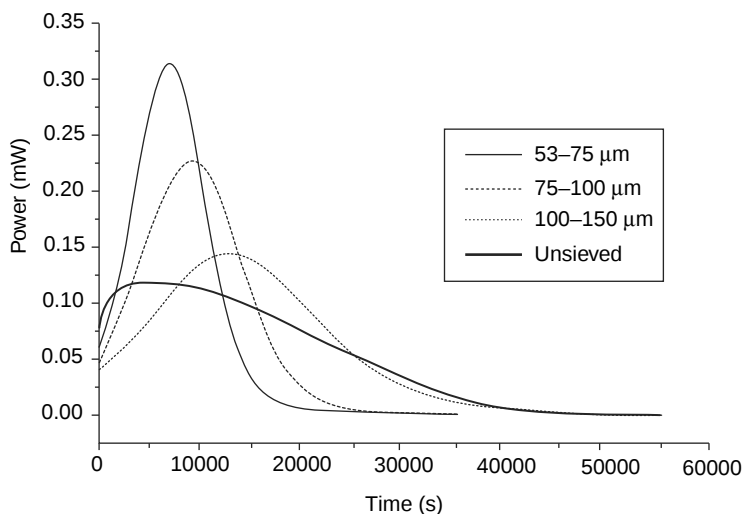


Figure 6 Conversion of seratrodist form II to form I at 58°C, 63% RH (43).

Table 4 Reaction Parameters for the Conversion of Seratrodist Form II to Form I at 58°C, 63% RH (43)

Particle Size (μm)	ΔH (kJ mol ⁻¹)	m	k (s ⁻¹)
53–75	-5.72	2.09	1.22×10^{-4}
75–100	-5.70	2.10	8.08×10^{-5}
100–150	-5.74	1.99	5.57×10^{-5}

Table 5 Stability Data for Seratrodist Form II to Form I Conversion as a Function of Temperature and Humidity (43)

Temp (°C)	RH (%)	k (s ⁻¹)	t_{90} (days)
25	13	3.01×10^{-10}	12493
25	31	3.11×10^{-10}	12070
25	63	9.65×10^{-10}	3893
25	93	3.84×10^{-9}	977
40	13	7.38×10^{-8}	50.9
40	31	7.94×10^{-8}	47.3
40	63	2.14×10^{-7}	17.5
40	93	9.30×10^{-7}	4.0

degradation reaction), to develop packaging materials that can ameliorate the effects of photodegradation and to estimate the *in vivo* photosensitizing potential of a drug from its *in vitro* photostability (photolabile drugs are often used for chemotherapy for instance). While many drugs decompose to some degree during exposure to light the practical consequences can be serious; nifedipine, an extremely photolabile drug, has a photochemical half-life of only a few minutes (44).

Until recently there were no established guidelines for photostability testing of drugs. ICH Guideline Q1b "Photostability testing of new drug substances and products" was adopted in 1997 and requires demonstration of no unacceptable change upon exposure of a compound to light, where "acceptable change" is change within limits justified by the applicant. This means that photocalorimetry already offers an excellent method by which to record photostability data and one that, in principle, has the scope also to produce quantitative kinetic data.

Typical instrument designs use optical light-guides (either fiber-optic cables or liquid-filled light guides) to introduce light into the ampoules. These enable the calorimetric cell to be fully enclosed in a thermostat and to be made of a single material. Schaarschmidt and Lamprecht (45) first described the use of light-guides in photocalorimetry in developing photocalorimeters for the study of living yeast cells. Cooper and Converse (46) transformed a batch calorimeter into a photocalorimeter by fitting it with quartz optical fiber light guides for their study of the photochemistry of rhodopsin.

Teixeira and Wadsö (47) developed a differential photocalorimeter using two twin calorimeters (i.e., employing four vessels; two samples with two corresponding references). Fiber-optic bundles guided light from a 100 W tungsten lamp through a monochromator before being split equally into the two sample vessels. One vessel, a stirred perfusion/titration vessel, was used for the measurement of the thermal power during a photochemical reaction. The other served as a photo-inert reference. The differential signal was recorded for each of the two twin calorimetric vessels.

The first use of photocalorimetry specifically for the assessment of pharmaceutical photostability was by Lehto et al. (48). Their apparatus consisted of a 75 W Xe-arc lamp which was used to introduce light through a grating monochromator via an assembly of focusing mirrors and a shutter. The light entry was split into two parts through two identical 1 mm optical light cables and introduced into the calorimetric vessels. As in the design discussed above, one of the vessels was used as a photocalorimetric vessel that recorded both the thermal activity of the photosensitive reaction and adsorption of the light while the other vessel served as a reference, recording solely the light adsorption. The instrument was used for photodegradation studies of photolabile compounds: nifedipine and L-ascorbic acid, at different wavelengths in solution and solid state.

Most recently, the development of a photocalorimeter for pharmaceutical photostability assessment has been pursued by Morris (49), Dhuna et al. (50,51), and Sousa et al. (52) using solid-state LEDs. The low power output of LEDs mean they can be incorporated directly into the calorimetric ampoule and, as long as the same light power is introduced to the reference cell, stable baselines can be achieved. Figure 7 shows the photodegradation of nifedipine in ethanolic solution at 25°C under a white-light LED (52). The data show a zero-order phase followed by a first-order decay as the nifedipine is exhausted. The baseline post-degradation allows the light power to be quantified.

STABILITY ASSESSMENT OF API-EXCIPIENT MIXTURES

There are few reported studies where IMC data of active-excipient blends have been analyzed quantitatively, usually because of the complexity of the data, although this forms perhaps the most exciting potential growth area for the use of IMC in the pharmaceutical sciences.

Using IMC it has been shown that under an RH of 100%, a binary mixture of aspirin and lactose shows no thermal response while a binary mixture of aspirin and magnesium stearate

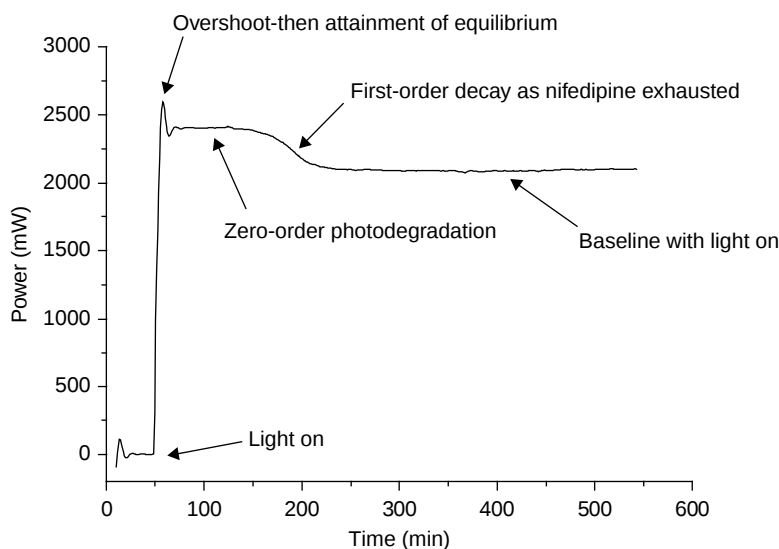


Figure 7 Photodegradation of nifedipine in ethanol solution with LED photocalorimetry (52).

shows a large exothermic signal (53). The heat output appeared to follow zero-order kinetics and lasted for several days. Analysis of the aspirin content in the ampoule after the power had returned to zero showed that the entire drug sample had degraded.

Interactions between a solid active and a range of excipients, including potato starch, α -lactose-monohydrate, microcrystalline cellulose (MCC), and talc have been investigated using IMC, albeit between elevated temperatures of 60–80°C (54). Large exothermic heat responses were observed for mixtures of drug with MCC, potato starch, and lactose, indicating that these systems were unstable. Similar studies have looked at the interactions of an active compressed into a tablet (55), and the effect of compression forces on the stability of dibasic calcium phosphate dihydrate in the presence of glutamic acid hydrochloride (56).

The stabilities of tablet formulations of enalapril maleate were studied with IMC (57) as a function of temperature and RH, with increases in both variables adversely affecting drug stability. Importantly, the IMC data correlated with classical stability data recorded with HPLC, indicating the potential power of IMC during formulation studies.

Flow-through microcalorimetry has been used to study the interaction between heparin sodium and dopamine hydrochloride in two parenteral formulations (58). A significant interaction between the drugs was noted when dextrose was included in the parenteral solution which was not seen in normal saline formulations.

The effect of menadione and prednisone on the physical stability of various microemulsions has been investigated by IMC (59). It was shown that neither drug influenced the stability of the formulation. Gaisford et al. (60) used IMC for studying the swelling of PEG-based hydrogels in water. In these experiments, a segment of dry hydrogel (xerogel) was immersed in water (1 mL) and the heat response from swelling recorded. A typical trace (Fig. 8) showed a two-phase process, which was ascribed to the hydration of the polymer core and subsequent relaxation of the polymeric network. The break point time between the two processes was observed to reduce with an increase in storage temperature.

More recently, the interaction between binary cationic lipids and DNA has been investigated with ITC (61). The data showed an endothermic binding between phosphate and ammonium groups distinct from an exothermic, kinetically slow formation of large, multilamellar liposome/pDNA complexes.

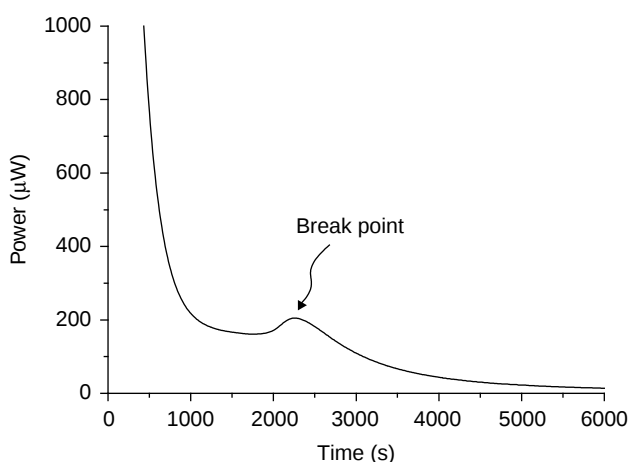


Figure 8 Power-time data for the swelling of a hydrogel segment, showing the break point between hydration of the gel and relaxation of the polymer network (60).

Table 6 Rate Constant Values for the Degradation of BPO in Combination with Various Excipients (62)

Excipient	k (s^{-1})
BPO alone	2.0×10^{-7}
2% polytrap	1.04×10^{-7}
1% sipernat	1.11×10^{-6}
0.15% monawet	3.40×10^{-8}
5% propylene glycol; 2.5% glycerine; 0.15% monawet	8.42×10^{-9}
5% propylene glycol; 2.5% glycerine; 2% polytrap	3.41×10^{-7}
Pluronic 234	7.24×10^{-9}

Stability Assessment of Formulated Products

The benefits of using IMC for stability assessment should be clear by this point, but the benefits for formulated products are perhaps greater than for any other sample. This is because of the ability of the technique simply to monitor the sample over time. The only issue facing the operator is to select a fraction of the sample for investigation that is representative of the whole (assuming the formulation in its entirety does not fit wholly within the calorimetric ampoule). However, applications in this area are not widespread in the literature, for many of the reasons previously raised in this chapter.

A good example of the use of IMC to quantify the stability of an active in a formulation is provided by Zaman et al. (62), who studied the degradation of benzoyl peroxide (BPO) with a number of excipients in an aqueous base. It was found that nearly all of the excipients commonly used to formulate BPO actually stabilized the system (data shown in Table 6); two of the excipients, Monawet and Pluronic 234, resulted in degradation rate constants two orders of magnitude below that seen for BPO alone. Only Sipernat was seen to decrease stability, which was ascribed to it being a Lewis acid.

The use of IMC to study crystallization in transdermal drug delivery systems (TDDS) has been reported for a number of actives (63–65). In these cases, 10 mm diameter disks were punched out of cast films and built up in layers in the calorimetric ampoule. The use of IMC for determining the stability of oral fast-dissolving films has also recently been discussed (66). Films of polyvinylpyrrolidone (PVP) containing indometacin were cast into glass ampoules;

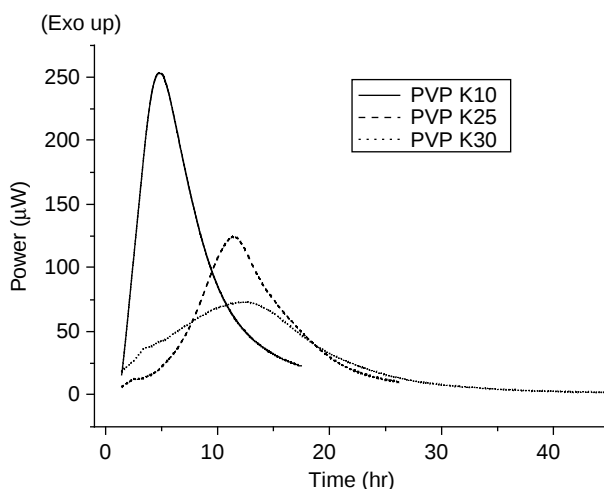


Figure 9 Power-time data observed for indometacin-PVP (all grades) films at 25°C (66).

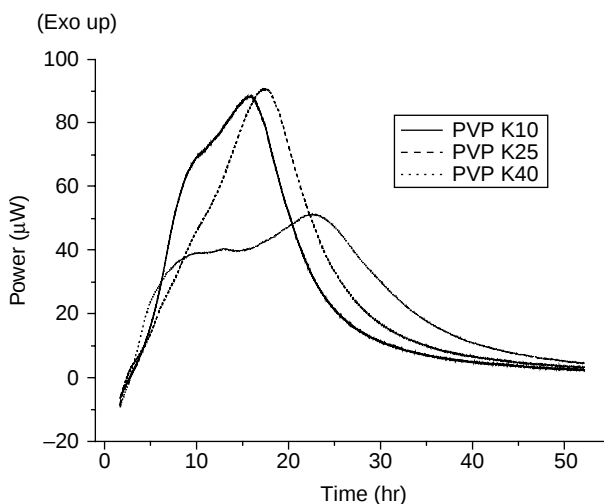


Figure 10 Power-time data observed for indometacin-PVP films (all grades) at 37°C (66).

stability was assessed by monitoring the power changes occurring with time. Three grades of PVP (K10, K25, and K40, where the number multiplied by 1000 gives the average molecular weight) were used. Indometacin was seen to crystallize from all PVP grades over ca. 24–48 hours at two study temperatures (25 and 37°C), as denoted by a large exothermic event. At 25°C the exothermic event was a single peak (Fig. 9); at 37°C two peaks were observed (Fig. 10). Subsequent analysis of the crystals with DSC and polarized light microscopy determined that the stable γ -polymorph of indometacin formed at 25°C while both the γ - and metastable α -polymorphs formed at 37°C. The calorimetric data were converted to relative crystallinity as a function of time (as described above) and analyzed with three crystallization models (Avrami, Tobin, and Urbanovici-Segal) to determine crystallization kinetics. The rate constants determined were broadly consistent irrespective of the model used. Increasing

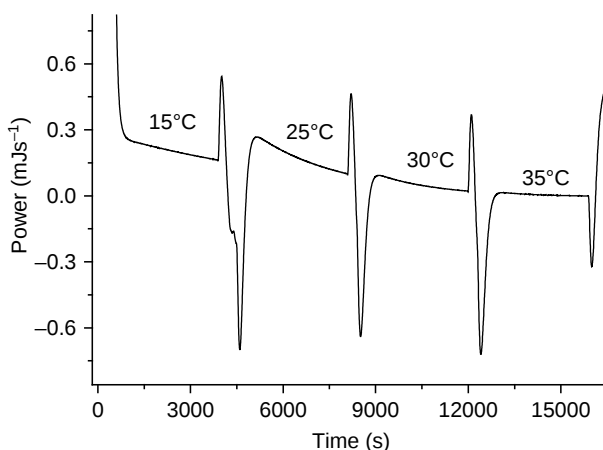


Figure 11 Power output obtained for the hydrolysis of methyl paraben during a step-isothermal DSC experiment (67).

polymer molecular weight did not generally affect the crystallization rate, although an increase in temperature did result in a concomitant increase in crystallization rate. The data suggested that isothermal calorimetry is capable of monitoring drug crystallization in polymer films and therefore the technique could be a useful tool for conducting stability assays for fast-dissolving oral medicines.

Step-Isothermal Studies

One benefit of DSC instruments, in particular, is their capacity rapidly to change temperature. If sufficient sample can be loaded into the ampoule, then it is possible to perform a step-isothermal experiment. Some DSC instruments are designed to hold relatively large volumes (up to 1 mL) of liquid or semi-solid samples. Here, the power is measured as the sample is held at a number of isothermal steps (say, 5°C increments). The capacity of the instrument to change temperature rapidly means that data at a number of temperatures can be recorded quickly and easily. Analysis of the data at each temperature to determine the rate constant allows construction of an Arrhenius plot and hence extrapolation of the stability profile to any other temperature. Such an approach was demonstrated by O'Neill et al. (67), using the hydrolysis of methyl paraben as a test reaction. Power data were recorded at four temperatures in a single experiment (Fig. 11). Because the hydrolysis was first order, plotting the data as $\ln(\text{power})$ versus time gave four linear regions, the slopes of which were the rate constants. Using these values to construct an Arrhenius plot resulted in a straight line.

A further use of step-isothermal experiments is for rapid screens of drug-excipient compatibility. An example is provided by the study of aspirin with two excipients using high-sensitivity DSC (HSDSC) (68). Figure 12 shows the HSDSC trace obtained for a binary mixture of acetylsalicylic acid (aspirin) with lactose, and Figure 13 shows the HSDSC trace obtained for a binary mixture of acetylsalicylic acid with magnesium stearate. In both cases, the samples have been subjected to the same temperature program (shown on the right-hand y-axis). It can be seen that at lower temperatures, there is no detectable heat flow in either of the mixtures. However, at 55°C, the acetylsalicylic acid/magnesium stearate mixture gives an endothermic signal. The aspirin/lactose mixture gives rise to no thermal signal over the course of the whole experiment. Such an observation suggests that acetylsalicylic acid is incompatible with magnesium stearate but not with lactose. Each experiment was completed in > 8 hours.

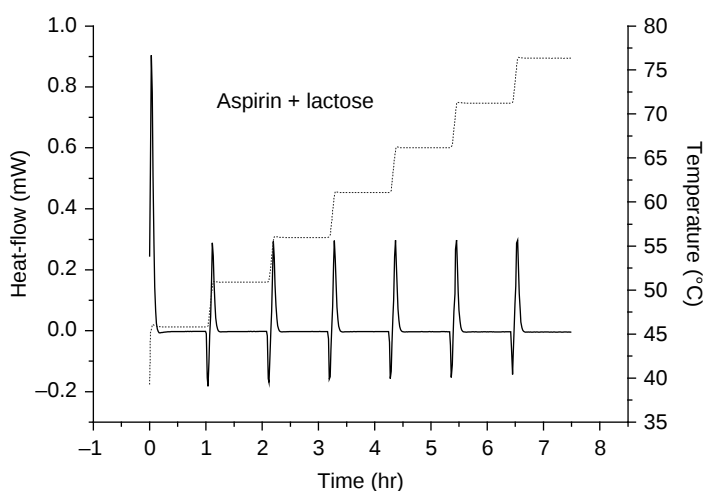


Figure 12 HSDSC trace obtained for a 1:1 mixture of aspirin:lactose over a programmed temperature ramp (68).

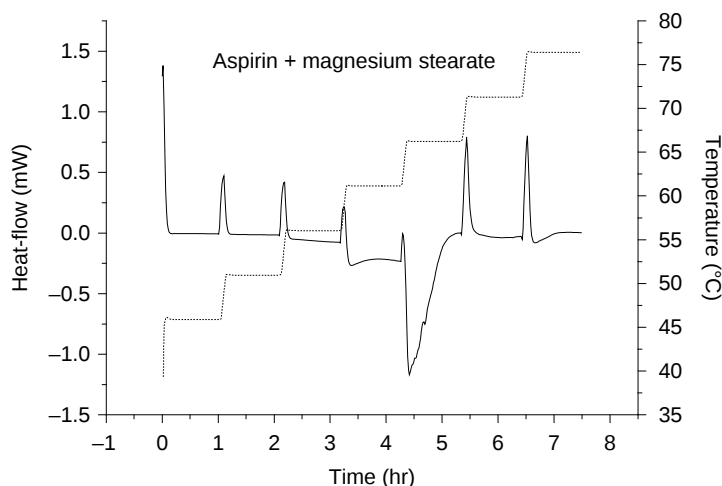


Figure 13 HSDSC trace obtained for a 1:1 mixture of aspirin:magnesium stearate over a programmed temperature ramp (68).

SUMMARY

IMC offers great potential for stress testing of pharmaceutical because it can be applied to virtually any sample. When characterizing the stability of individual pharmaceuticals, the methodology is relatively simple, assuming that any degradation pathway is not overly complex, and it is easily possible to determine degradation rate constants. The use of the technique for stress testing formulated materials is not so widely applied, despite its considerable advantage of being able to study whole samples without any purification steps. Several potential reasons for this present themselves. Firstly, the data recorded may be complex, containing contributions from both chemical and physical changes of multiple components. Secondly, medicines are formulated with acceptable stability over many years; hence, little degradation would be expected in a formulated product. The challenge facing the user of any analytical technique is,

therefore, to make a judgment as to whether a zero signal means there is no change in the sample material or the magnitude of any change is below the detection level of the instrument. Finally, IMC data give no mechanistic insight into the process under study and IMC data in isolation are not currently accepted in regulatory documents.

Developments in data analysis routines and experimental design can address the first issue, while improvements in sensitivity give the confidence to make sound judgments for the second issue. Acceptance of IMC data in regulatory submissions will only come through publication of IMC data and comparison of the results with classical analytical techniques (such as chromatography and X-ray diffraction); this then forms what is perhaps the most pressing area for current research efforts and academic-industrial collaboration.

FURTHER INFORMATION

Although there are a number of excellent books that provide information on pharmaceutical thermal analysis, many do not include sections on isothermal calorimetry. Specialist texts include *Biological Microcalorimetry*, edited by AE Beezer, Academic Press (London), 1982 and *Pharmaceutical Isothermal Calorimetry*, by S Gaisford and MAA O'Neill (Informa Healthcare), 2006, while an excellent chapter can be found in *Principles of Thermal Analysis and Calorimetry*, edited by PJ Haines, RSC (Cambridge), 2002. Aspects of isothermal titration calorimetry are covered in two texts; *Biocalorimetry*, edited by JE Ladbury and BZ Chowdhry, Wiley (Chichester), 1998 and *Biocalorimetry II*, edited by JE Ladbury and ML Doyle, Wiley (Chichester), 2004.

REFERENCES

1. Cooper A, Johnson CM, Lakey JH, et al. Heat does not come in different colours: entropy–enthalpy compensation, free energy windows, quantum confinement, pressure perturbation calorimetry, solvation and the multiple causes of heat capacity effects in biomolecular interactions. *Biophys Chem* 2001, 93: 215–30.
2. Wadsö I. Calculation methods in reaction calorimetry. *Science Tools: The LKB Instr J* 1966, 13: 33–9.
3. G Buckton, AE Beezer. The applications of microcalorimetry in the field of physical pharmacy. *Int J Pharm* 1991, 72: 181–91.
4. AE Beezer, S Gaisford, AK Hills, RJ Willson, JC Mitchell. Pharmaceutical microcalorimetry: Applications to long term stability studies. *Int J Pharm* 1999, 179: 39–45.
5. Wadsö I. Recent developments in reaction and solution calorimetry. *Thermochimica Acta* 1990, 169: 151–60.
6. Hansen LD. Instrument selection for calorimetric drug stability studies. *Pharm Tech* 1996, 20: 64–74.
7. Souillac PO, Dave P, Rytting JH. The use of solution calorimetry with micellar solvent systems for the detection of polymorphism. *Int J Pharm* 2002, 231: 185–96.
8. Willson RJ, Sokoloski TD. Ranking of polymorph stability for a pharmaceutical drug using the Noyes–Whitney titration template method. *Thermochimica Acta* 2004, 417: 239–43.
9. Arnot LF, Minet A, Patel N, et al. Solution calorimetry as a tool for investigating drug interaction with intestinal fluid. *Thermochimica Acta* 2004, 419: 259–66.
10. Chadha R, Kashid N, Jain DVS. Microcalorimetric evaluation of the in vitro compatibility of amoxicillin/clavulanic acid and ampicillin/sulbactam with ciprofloxacin. *J Pharm Biomed Anal* 2004, 36: 295–307.
11. Chadha R, Kashid N, Kumar A, et al. Calorimetric studies of diclofenac sodium in aqueous solution of cyclodextrin and water–ethanol mixtures. *J Pharm Pharmacol* 2002, 54: 481–6.
12. Harjunen P, Lehto V-P, Koivisto M, et al. Determination of amorphous content of lactose samples by solution calorimetry. *Drug Dev Ind Pharm* 2004, 30: 809–15.
13. Hogan SE, Buckton G. The quantification of small degrees of disorder in lactose using solution calorimetry. *Int J Pharm* 2000, 207: 57–64.
14. Barriocanal L, Taylor KMG, Buckton G. A study of liposome formation using a solution (isoperibol) calorimeter. *Int J Pharm* 2004, 287: 113–21.
15. Hansen LD, Eatough DJ. Comparison of the detection limits of microcalorimeters. *Thermochimica Acta* 1983, 70: 257–68.
16. Greenspan L. Humidity fixed points of binary saturated aqueous solutions. *J Res Nat Bur Standards* 1977, 81A: 89–96.

17. Nyqvist H. Saturated salt solutions for maintaining specified relative humidities. *Int J Pharm Tech & Prod Mfr* 1993, 4: 47–8.
18. Kommanaboyina B, Rhodes CT. Trends in stability testing, with emphasis on stability during distribution and storage. *Drug Dev Ind Pharm* 1999, 25: 857–68.
19. Willson RJ. *PhD Thesis*, University of Kent at Canterbury, 1995.
20. Pikal MJ. Results of evaluation of the LKB2277 calorimeter for stability testing of pharmaceuticals. Application Note 335, Thermometric AB, Järfälla, Sweden, 1983.
21. Phipps MA, Winnike RA, Long ST, et al. Excipient compatibility as assessed by isothermal microcalorimetry. *J Pharm Pharmacol* 1998, 50(S9).
22. Phipps MA, Mackin LA. Application of isothermal microcalorimetry in solid state drug development. *PSTT* 2000, 3: 9–17.
23. Rowe RC, Parker MD, Bray D. Batch and source variations in excipients – quantification using microcalorimetry. *Pharm Tech Eur* 1994, Feb: 26–30.
24. Schmitt EA, Peck K, Sun Y, et al. Rapid, practical and predictive excipient compatibility screening using isothermal microcalorimetry. *Thermochimica Acta* 380 2001: 175–83.
25. Schmitt EA. Excipient compatibility screening by isothermal calorimetry. 53rd Calorimetry Conference, August 9–14, Midland, Michigan, USA, 1998.
26. Buckton G. Applications of isothermal microcalorimetry in the pharmaceutical sciences. *Thermochimica Acta* 1995, 248: 117–29.
27. Gaisford S, Buckton G. Potential applications of microcalorimetry for the study of physical processes in pharmaceuticals. *Thermochimica Acta* 2001, 380: 185–98.
28. The Pharmaceutical Codex, 12th edn. London: Pharmaceutical Press, 1994.
29. Koenigbauer MJ, Brooks SH, Rullo G, et al. Solid-state stability testing of drugs by isothermal calorimetry. *Pharm Res* 1992, 9: 939–44.
30. Hansen LD, Eatough DJ, Lewis EA, et al. Shelf-life prediction from induction period calorimetric measurements on materials undergoing autocatalytic decomposition. *Can J Chem* 1990, 68: 2111–14.
31. Otsuka T, Yoshioka S, Aso Y, et al. Application of microcalorimetry to stability testing of meclofenoxate hydrochloride and *dl*- α -tocopherol. *Chem Pharm Bull* 1994, 42: 130–2.
32. Oliyai R, Lindenbaum S. Stability testing of pharmaceuticals by isothermal heat conduction calorimetry: ampicillin in aqueous solution. *Int J Pharm* 1991, 73: 33–6.
33. Pikal MJ, Dellerman KM. Stability testing of pharmaceuticals by high-sensitivity isothermal calorimetry at 25°C: cephalosporins in the solid and aqueous states. *Int J Pharm* 1989, 50: 233–52.
34. Chada R, Kashid N, Jain DVS. Kinetics of degradation of diclofenac sodium in aqueous solution determined by a calorimetric method. *Pharmazie* 2003, 58: 631–5.
35. Simoncic Z, Rokar, R, Gartner A, et al. The use of microcalorimetry and HPLC for the determination of degradation kinetics and thermodynamic parameters of perindopril erbumine in aqueous solutions. *Int J Pharm* 2008, 356: 200–5.
36. Angberg M, Nyström C. Evaluation of heat-conduction microcalorimetry in pharmaceutical stability studies: I. Precision and accuracy for static experiments in glass vials. *Acta Pharm Suec* 1988, 25: 307–20.
37. Angberg M, Nyström C, Castensson S. Evaluation of heat-conduction microcalorimetry in pharmaceutical stability studies: II. Methods to evaluate the microcalorimetric response. *Int J Pharm* 1990, 61: 67–77.
38. Skaria CV, Gaisford S. 2005. (unpublished data).
39. Skaria CV, Gaisford S, O'Neill MAA, et al. Stability assessment of pharmaceuticals by isothermal calorimetry: two component systems. *Int J Pharm* 2005, 292: 127–35.
40. Tan X, Meltzer N, Lindenbaum S. Solid-state stability studies of 13-*cis*-retinoic acid and all-*trans*-retinoic acid using microcalorimetry and HPLC analysis. *Pharm Res* 1992, 9: 1203–8.
41. Hansen LD, Lewis EA, Eatough DJ, et al. Kinetics of drug decomposition by heat conduction calorimetry. *Pharm Res* 1989, 6: 20–7.
42. Lechuga-Ballesteros D, Bakri A, Miller DP. Microcalorimetric measurement of the interactions between water vapour and amorphous pharmaceutical solids. *Pharm Res* 2003, 20: 308–18.
43. Urakami K, Beezer AE. A kinetic and thermodynamic study of seratrodoast polymorphic transition by isothermal microcalorimetry. *Int J Pharm* 2003, 257: 265–71.
44. Thoma K, Klimek R. Photostabilization of drugs in dosage forms without protection from packaging and materials. *Int J Pharm* 1991, 67: 169–75.
45. Schaarschmidt B, Lamprecht I. UV-irradiation and measuring of the optical density of microorganisms in a microcalorimeter. *Experientia* 1973, 29: 505–6.

46. Cooper A, Converse CA. Energetics of primary process in visual excitation: photocalorimetry of rhodopsin in rod outer segment membranes. *Biochem* 1976, 15: 2970–8.
47. Teixeira C, Wadsö I. A microcalorimetric system for photochemical processes in solution. *J Chem Thermodyn* 1990, 22: 703–13.
48. Lehto VP, Salonen J, Laine E. Real time detection of photoreactivity in pharmaceutical solids and solutions with isothermal microcalorimetry. *Pharm Res* 1999, 16: 368–73.
49. Morris AC. PhD Dissertation, University of Greenwich, 2004.
50. Dhuna M, Beezer AE, Morris AC, et al. Development of an isothermal heat-conduction photocalorimeter. *Rev Sci Instr* 2007, 78: (025105)1–5.
51. Dhuna M, Beezer AE, Connor JA, et al. LED-array photocalorimetry: instrument design and application to photostability of nifedipine. *J Pharm Biomed Anal* 2008, 48: 1316–20.
52. Sousa L, Hansen, LD, Gaisford S, et al. 2010. (unpublished data).
53. Potluri K. MSc Dissertation, University of London, 2003.
54. Selzer T, Radau M, Kreuter J. Use of isothermal heat-conduction microcalorimetry to evaluate stability and excipient compatibility of a solid drug. *Int J Pharm* 1998, 171: 227–41.
55. Selzer T, Radau M, Kreuter J. The use of isothermal heat conduction microcalorimetry to evaluate drug stability in tablets. *Int J Pharm* 1999, 184: 199–206.
56. Al-Hallak MHDK, Xu ZH, Ghaffari F, et al. The effect of compression forces on the stability of dibasic calcium phosphate dihydrate tablets in the presence of glutamic acid hydrochloride monitored by isothermal calorimetry. *Thermochimica Acta* 2008, 467: 86–90.
57. Simoncic Z, Zupancic P, Roskar R, et al. Use of microcalorimetry in determination of stability of enalapril maleate and enalapril maleate tablet formulations. *Int J Pharm* 2007, 342: 145–51.
58. Pereira-Rosario R, Utamura T, Perrin JH. Interaction of heparin sodium and dopamine hydrochloride in admixtures studied by microcalorimetry. *Am J Hosp Pharm* 1988, 45: 1350–2.
59. Fubini B, Gasco MR, Gallarate M. Microcalorimetric study of microemulsions as potential drug delivery systems: 2. Evaluation of enthalpy in the presence of drugs. *Int J Pharm* 1989, 50: 213–17.
60. Gaisford S, Buckton G, Forsyth W, et al. Isothermal microcalorimetry as a tool to investigate the swelling and drug release of a PEG-based hydrogel. *J Pharm Pharmacol* 2000, 52(S): 304.
61. Giatrellis S, Nikolopoulos G, Sideratou Z, et al. Calorimetric study of the interaction of binary DMTAP/DOTAP cationic liposomes with plasmid DNA. *J Liposome Res* 2009, 19: 220–30.
62. Zaman F, Beezer AE, Mitchell JC, et al. The stability of benzoyl peroxide formulations determined from isothermal microcalorimetric studies. *Int J Pharm* 2001, 225: 135–43.
63. Latsch S, Selzer T, Fink L, et al. Crystallisation of estradiol containing TDDS determined by isothermal microcalorimetry, X-ray diffraction and optical microscopy. *Eur J Pharm Biopharm* 2003, 56: 43–52.
64. Latsch S, Selzer T, Fink L, et al. Determination of the physical state of norethindrone acetate containing transdermal drug delivery systems by isothermal microcalorimetry, X-ray diffraction and optical microscopy. *Eur J Pharm Biopharm* 2004, 57: 383–95.
65. Latsch S, Selzer T, Fink L, et al. Use of isothermal microcalorimetry, X-ray diffraction and optical microscopy for characterisation of crystals grown in steroid-containing transdermal drug delivery systems. *Eur J Pharm Biopharm* 2004, 57: 397–410.
66. Gaisford S, Verma A, Saunders M, et al. Monitoring crystallisation of drugs from oral fast-dissolving films with isothermal calorimetry. *Int J Pharm* 2009, 380: 105–11.
67. O'Neill MAA, Gaisford S, Beezer AE, Skaria CV, Sears P. Application of a test and reference reaction. *J Therm Anal Cal* 2006, 84: 301–6.
68. Wissing S, Craig DQM, Barker SA, Moore WD. An investigation into the use of stepwise isothermal high sensitivity DSC as a means of detecting drug-excipient compatibility. *Int J Pharm* 2000, 199: 141–50.

23 | Temperature excursions during shipment and storage

Manuel Zahn

INTRODUCTION

Stress testing both on the drug substance and drug product complements the formal stability studies for registration and the knowledge gained from GMP maintenance studies (on-going stability studies). All data generated during development through to the marketing phase contribute to the database available for stability assessment. Although the ICH Stability Guideline Q1A does not require systematic stress tests on the drug product except photostability tests, some short-term tests on one batch are highly recommended (1) as part of development in order to understand the impact of high temperature on the product that could be encountered during shipment and storage. The result of these tests facilitates the evaluation of the temperature monitoring data and the decision as to whether a particular batch has to be withdrawn from the distribution chain.

BUILDING A SOLID FOUNDATION

The first step toward developing a science-based approach to support product distribution has been well documented in this book, and that is to perform forced stressed degradation studies to identify the “potential” routes of degradation that a drug molecule may follow. The core set of stresses commonly used is summarized in Table 1 and focuses largely on the degradation of the API, to determine when the drug is fully solubilized (and accessible to react) what types of chemistry may occur. In these studies, one presents a maximized exposure of the drug molecule that then allows the assessment of the susceptibility of the drug to these degradation chemistries.

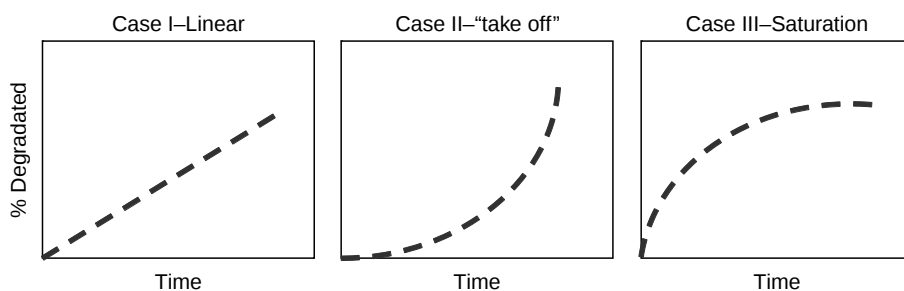
After establishing what types of chemistries can occur, one must translate it back to either the drug substance or the drug product to determine those that are relevant under expected storage and exposure conditions. For example, the drug substance material is left as a physical solid (i.e., undissolved state), and then stressed to find out, which of the chemistries identified above are actually observed in the packaged sample. The key parameters investigated here are temperature and humidity and may include open dish (i.e., constant temperature and humidity studies) to understand how moisture affects the stability of the drug product, and—as a consequence—is there a need for a desiccant. The crystal habit of the drug substance begins to then influence which chemistries occur, and to what extent. For example, a hypothetical drug substance that when fully solubilized undergoes significant oxidative degradation, but in a robust crystal habit does not show any evidence of oxidative degradation in the drug product.

On the other hand, a drug product can be heavily affected by the types of excipients that now provide a new “solvent” for the drug substance, whether the surrounding excipients are solids (e.g., a tablet) or liquids (e.g., a liquid-filled capsule or parenteral product), that adds reactive components to the mix, and thus influences the types of chemistries observed. Most often, these chemistries are a subset of the results determined from the testing in Table 1; however, sometimes they are new (e.g., an adduct that is formed with an excipient). Furthermore, the manufacturing process for the drug product can add solvents, heat, create regions of amorphous drug, open new crystal faces that are more reactive, etc., which can also frame the extent and type of chemistries that one observes.

Depending on the nature of these reactions in drug substance or drug product, the profile for the amount of degradation over time can take varying shapes. Three common examples are outlined in Figure 1.

Table 1 Core Set of Forced Stress Testing Studies to Understand Types and Extents of Degradation of a Drug Molecule (See chap. 2 for Additional Details)

Acid
Base
Heat (5–80°C); humidity (20–75%)
ROO•-mediated oxidation
Fe-mediated oxidation
Cu-mediated oxidation
Peroxide-mediated oxidation
Photostress

**Figure 1** Typical degradation—time profiles for drug substances and drug products.

Linear degradation-time profiles (Case I in Fig. 1) are typically observed for chemistries that do not require “reagents” for the chemistry to occur, or the “reagent” is not limiting, and the chemistry occurs in a predictable manner. Often, the rate of degradation follows well-established trends as a function of temperature (e.g., Arrhenius behavior, *vide supra*) and thus maximizes the long-term predictability of the degradation trend.

“Take off” degradation-time profiles (Case II in Fig. 1) are typically observed for chemistries that require an “induction” period for the reaction. For example, many oxidative reactions are auto-catalytic, and thus reactive reagents (e.g., ROOH moieties) are built up over time, leading to increased rates and extent of reaction. Another example of increased reagent formation is the production of formaldehyde or formic acid that results from oxidative degradations of some excipients (e.g., PEG). Obviously, “take off” degradation-time profiles are the most difficult when it comes to long-term predictability, and often real time data at a given storage condition is the favored choice for shelf-life prediction.

Finally, saturation degradation-time profiles (Case III, Fig. 1) are often observed and can frequently be attributed to the consumption of small levels of impurities (e.g., solvent, reactive species) or consumption of small amounts of reactive drug environments (e.g., amorphous or crystal fracture). In these cases, as the reactive components are consumed, the chemistry stops occurring, and the profile for both the degradation product and assay amounts level off. Saturation degradation-time profiles are often very amenable to long-term predictability, in that all temperature storage conditions evolve to the same plateau value, but just at different rates (see Fig. 2, as an example). Such differences in degradation-time profiles can be taken into account by using an “isoconversion” approach, as demonstrated by the accelerated stability assessment protocol (2), where degradation kinetics are predicted using a humidity-corrected Arrhenius equation and the total degradation for each condition is set to provide approximately the same amount of degradation.

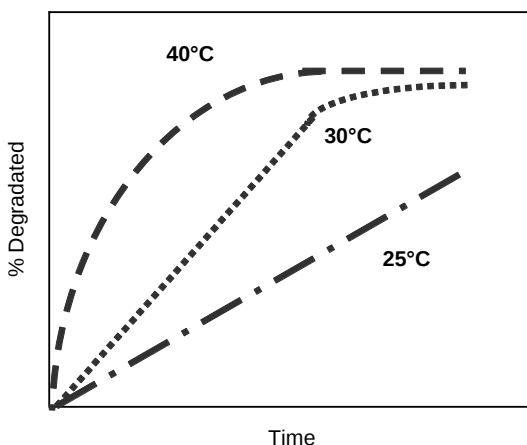


Figure 2 Impact on temperature for saturation degradation-time profiles.

Typically, early in development (clinical phase I to early phase III), accelerated stability conditions are used to establish shelf-lives for API and drug product used in clinical studies, and rely on developing a good understanding of the overall shape of the degradation profile (i.e., Case I, Case II, or Case III in Fig. 1). However, prior to registration of a new product, where also now the API and the drug product processes are more established and representative of commercial scale, the focus turns to establishing a stability profile that can be used to support shelf-life establishment for commercial production. A stability study is typically performed at several temperatures as a function of time with the commercial representative product, to then firmly establish the shape of the degradation-time profile, and hopefully confirming what was already understood during the phase I–III stability studies.

The key focus of this chapter are stability profiles that are shown to exhibit linear time-degradation profiles (e.g., Case I, Fig. 1), as these examples are most amenable to mathematical treatments to predict long-term shelf-life expectations with ambient storage from short-term accelerated data. Moreover, well-behaved saturation time-degradation profiles may also lend themselves to rate-dependent treatments as a function of storage temperature, and may also find application of mathematical models outlined here in this chapter.

TESTING CONDITIONS TO SUPPORT STORAGE AND SHIPMENT

A typical stability program for a stable pharmaceutical product intended to be marketed worldwide is listed in Table 2.

A similar design could easily be developed for temperature-sensitive pharmaceuticals like vaccines or biologics using appropriate lower temperatures. The result of these tests conducted at three different temperatures or at least two offers the possibility of calculating the chemical reaction rate (k), that is, the rate of decomposition at each temperature, the activation energy (E_a) of the particular product in a particular pack, as well as the pre-exponential factor A in the Arrhenius equation.

ARRHENIUS EQUATION

In 1903, the Swedish chemist Svente Arrhenius was granted the Nobel Prize for the development of an equation that allows calculating the increase of the rate of chemical reactions with increasing temperature:

$$k = Ae^{-E_a/RT}$$

where
 k = rate constant
 A = pre-exponential factor
 E_a = activation energy
 R = universal gas constant = 8.314 (J/°K mol)
 T = temperature (°K).

In the case of pharmaceutical products, the aim is to slow down the chemical reaction, that is, the degradation of the active ingredient(s) or the increase of degradation products. Nevertheless, the Arrhenius equation is most applicable when the time-degradation profile is linear for all temperature studies, and care is taken to avoid crossing “physical transitions” of the API (e.g., melting point, dehydration, etc.) or the excipients used for the drug product (e.g., glassy to amorphous transition, etc.). It is also recognized that reaction rates can be extracted from degradation time profiles other than Case I (Fig. 1) examples, where the plot of $\ln k$ versus $1/T$ is found to be linear, and thus can also be assessed using the relationship above.

For Case I (linear time-degradation profiles), the concentration of the drug substance or the increase of known degradation products can be plotted over time (t), as shown in Figure 3.

Table 2 Global Stability Testing Program

	Testing Time (Months)								
Testing conditions	0	1	3	6	9	12	18	24	36
25°C/60% RH ^c long-term for CZ ^d I and II	+		+ ^a	+	+	+	+	+	+
30°C/65% RH intermediate	+		(+) ^b	(+)	(+)	(+)			
30°C/75% RH long-term for CZ IVB	+		+	+	+	+	+	+	+
40°C/75% RH accelerated	+	(+)	+	+					
50°C/ambient humidity stress test	+	+	(+)						

^a+: Samples tested
^b(+): Samples stored but may not be tested
^cRH: Relative humidity
^dCZ: Climatic zone

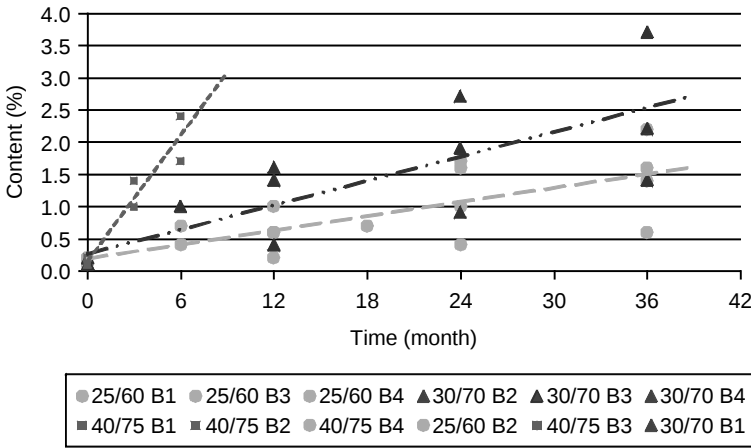


Figure 3 Increase of degradation products in three different batches over time at different temperatures.

As expected, the degradation is faster at higher temperatures. Also, the slope of the linear regression line for each temperature gives the rate constant k , same as in the Arrhenius equation at each temperature used in the stability study. A stepwise approach is recommended as follows:

1. Consider the order of reaction (assumed to be of zero order, which is applicable in the case of stable products);
2. Run a regression analysis of the values found for each temperature separately (linear regression);
3. The slope of the regression line provides the *constant* k for each temperature tested.

ARRHENIUS PLOT

If at the next step the different values for $\ln k$ are plotted versus the temperature ($1/T$), the so-called Arrhenius plot is achieved, as illustrated in Figure 4.

This plot provides other elements of the Arrhenius equation, namely the formulation-specific *activation energy* E_a and the pre-exponential factor A . While the slope of the line in the Arrhenius plot is equal to E_a/R , the intercept is equal to $\ln A$. As a consequence, the mean kinetic temperature (MKT) can be calculated (see below) based on product-specific factors. The Arrhenius plot can also be used to calculate each degradation rate constant k at any given temperature, preferably within the dataset rather than by extrapolation. A well-established temperature range, for example, that includes temperatures that are anticipated during product shipping, is particularly helpful in cases where the temperatures during shipment are monitored, and the MKT is calculated for the shipment period using actual values for E_a instead of an estimated average activation energy of $E_a = 83.144 \text{ kJ/mol}$ that is mentioned in the USP. The Arrhenius plot can also be developed based on short-term stress testing data generated during relevant forced stress studies by applying ASAP (3).

MEAN KINETIC TEMPERATURE

The MKT includes the reaction rate constants in the evaluation of the impact of heat on pharmaceutical products. A suitable definition of the MKT is as follows:

The MKT is the temperature corresponding to the effects of a given temperature-time distribution on chemical reaction kinetics.

The MKT allows calculating the impact of temperature fluctuations on the chemical degradation of a substance in a given product.

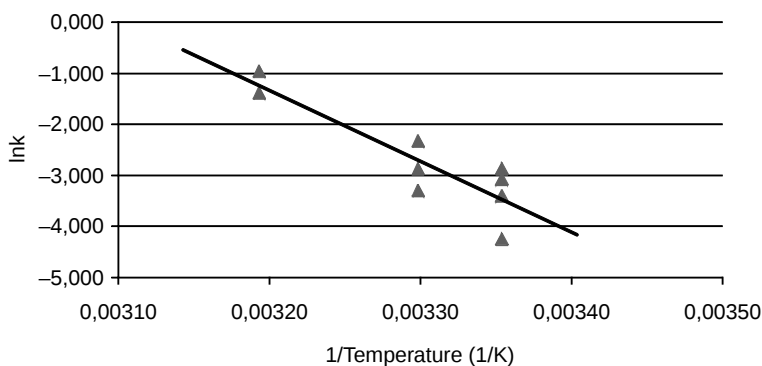


Figure 4 Arrhenius plot calculated using the degradation rates at three different temperatures.

The MKT can be calculated by using the formula developed by Haynes based on the Arrhenius equation (4):

$$MKT = \frac{E_a/R}{-\ln \frac{e^{-E_a/RT_1} + e^{-E_a/RT_2} + \dots}{n}}$$

where,

MKT = mean kinetic temperature (°K)

E_a = Activation energy (kJ/mol)

R = Universal gas constant = 8.314 (J/°K mol)

T = Temperature (°K)

n = Number of time points.

If not known, the activation energy E_a is assumed to be 83.144 kJ/mol. This value, which is recommended in the U.S. Pharmacopeia, has been derived from evaluating published data for more than 100 chemical substances, that is, small molecules that are commonly used as active ingredients in pharmaceutical products, and calculating the mean (5). If feasible and definitely in the case of biological/biotech products, it is always preferred to use the actual activation energy found for the particular substance instead of the USP recommended value. The actual activation energy can be derived by calculating the Arrhenius plot as described above.

The activation energy E_a (either calculated or assumed to be 83.144 kJ/mol) is divided by the universal gas constant R (0.00831432 kJ/°K mol).

The result is then divided by the temperature T_n (measured in degrees kelvin) to get a factor f_n for each time point n :

$$f_n = e^{-(E_a/R)/T_n}$$

After that, the sum of the individual results for a defined time period is divided by n , the number of time points used.

$$F_n = (f_1 + f_2 + \dots + f_n)/n$$

Then, the MKT (°K) for a defined time period is achieved by calculating the negative natural logarithm of the above result using the following equation:

$$MKT = (E_a/R)/(-\ln F_n)$$

The MKT is converted into degrees Celsius by subtracting 273.15 from the value found.

This enables the manufacturer to calculate the amount of heat energy over a defined time period, e.g., the time during storage in a warehouse or during shipment (see below). Companies providing temperature monitoring devices offer computer programs that calculate the MKT by taking the temperature measured every 5 or 10 minutes. Monitoring of the humidity during shipment of packed pharmaceutical products is usually not necessary. The time is normally too short, and the water vapor transmission too slow to let the changing humidity of the environment during shipping to have an impact on the stability of the packed products.

ASSUMPTIONS

In the approach described above, the following assumptions have been made.

- Linear Arrhenius plot, that is, an extrapolation may be possible.
- No change of mechanism.
- No change of molecularity of reaction.
- The activation energy E_a and the Arrhenius factor A are not temperature dependent.
- The degradation is linear: $c = c_0 - k \cdot t$.

These assumptions are usually valid for degradation in the order of 10–20% (6).

DEGRADATION DURING STORAGE AND SHIPMENT

Stability data—either generated by stress tests or at standard testing conditions – are the prerequisite to calculate the expected remaining shelf-life for a particular batch of a pharmaceutical substance or product after a certain period of storage and shipment.

Three different phases can be distinguished in the lifetime of a product (see Fig. 5):

1. *Storage* in the warehouse at the production site, normally at a controlled temperature.
2. *Shipment* from the production site to the wholesaler in the target country.
3. *Distribution* to the hospital, the pharmacy, the medical doctor, or the patient.

As the amount of active substance in the pharmaceutical product at time of release after production is known [c_0] as well as the time between production and start of shipment, and the degradation rate constant k at the long-term testing temperature, the following equation can be used to calculate the remaining assay at the end of the first phase:

$$c_{\text{storage}} = c_0 - k \cdot t$$

c_0 = initial assay at t_0 (%)

c_{storage} = assay after storage (%)

$k = k$ at long-term testing temperature or at the actual MKT

t = storage time.

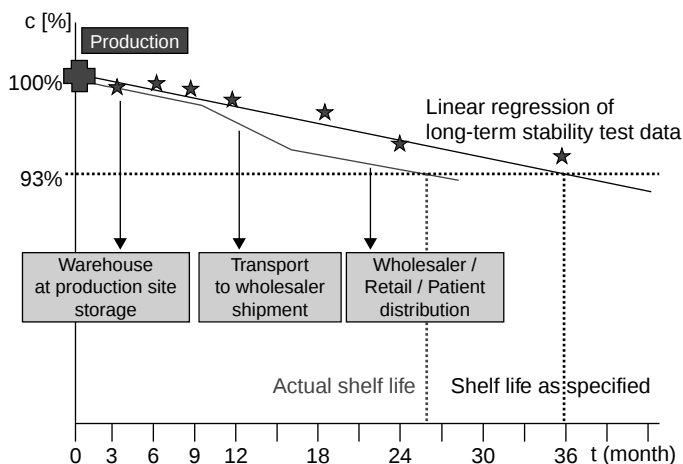


Figure 5 Degradation of active substance in a pharmaceutical product during storage, shipment, and distribution.

If the temperature during storage is monitored, the actual MKT can be used, which may be lower than the long-term stability testing condition, if the assumption that the degradation follows zero-order kinetics is valid, which is often the case, as described above. Furthermore, the validity of this assumption can be extracted from accelerated stress data that was generated to support product development. The remaining amount of active substance after shipment can be calculated using the following equation, provided that the temperature has been monitored during shipment:

$$C_{\text{shipment}} = C_{\text{storage}} + k \cdot t$$

C_{shipment} = assay after shipment (%)

C_{storage} = assay after storage (%)

k = k at calculated MKT

t = shipment time.

For the last phase, the equation looks like this:

$$C_{\text{distribution}} = C_{\text{shipment}} + k \cdot t$$

C_{shipment} = assay after shipment (%)

$C_{\text{distribution}}$ = assay after distribution (%)

k = k at long-term testing temperature

t = remaining shelf-life.

A 95% confidence interval is also recommended to produce a comfortable margin of error for the distribution phase, as outlined in the ICH guideline Q1E, instead of just using the linear regression line to calculate the remaining amount of assay or degradation product at the end of the shelf-life. If the calculated value for c at the expiry date for a particular product is less (in the case of assay) or higher (in the case of known degradation products) than respective specified acceptance criteria, the packs have to be withdrawn from the market. Otherwise, the product can be expected to be in compliance with the acceptance criteria until the end of the shelf-life.

LIMITATION TO THE USE OF ARRHENIUS AND MKT CALCULATIONS

The MKT approach has limitations that have to be observed when the impact of temperature on the stability of an active substance or product is being evaluated. The most important restriction is the fact that MKT only addresses chemical degradation. A substance, and in particular a pharmaceutical product, has also to meet other quality parameters within specified acceptance criteria throughout its shelf-life. A typical example is a suppository that is not allowed to be transported or stored above 30°C. Another example is cyclophosphamide monohydrate that melts at 49.5°C and is freely soluble in water. Short-term storage above 50°C converts the active substance to the anhydrous form that forms a cake with a slow dissolution rate (7).

Also at higher temperatures, if the mechanism of the chemical degradation changes or no longer follows zero- or first-order kinetics, the Arrhenius equation would not be applicable. The impact of high or low humidity on the physico-chemical stability of a packed product can normally be neglected for the short-term shipment period that last no longer than a few days. Some pharmaceutical forms, however, may be affected, e.g., gelatin capsules may show cracks at low humidity, effervescent tablets may start to dissolve at high humidity, and prolonged-release oral dosage forms may release the active substance faster than specified when the packaging material is not sufficiently protective.

REGULATORY REQUIREMENTS

It goes without saying that calculations as described above cannot be conducted without data, in particular, without monitoring of the temperature during storage and shipment. Temperature monitoring is thus, for good reasons, required by several regulations and guidelines valid in different countries and regions. In the following paragraph, the most relevant requirements are summarized.

Under US laws and regulations, it is prohibited to introduce an adulterated drug to the market, adulterate a drug, or receive a drug that is adulterated (8). A drug is regarded as adulterated if the facilities or controls used for its manufacture, processing, packing, or holding do not conform to or are not operated or administered in conformity with current good manufacturing practices (cGMP). Holding of a drug in this context includes distribution, transportation, and storage. In other words, storage and shipment of pharmaceuticals have to be done in line with cGMP that require monitoring the temperature.

According to a Canadian guideline (9) “drugs must be stored, handled, and transported according to storage conditions, e.g., temperature and humidity, specified on the label.”

Several GMP and Good Distribution Practice (GDP)-related guidelines have to be observed by manufacturers, e.g., the pharmacopoeial requirements for shipment and storage of pharmaceuticals as described in the United States Pharmacopeia (10) and in a World Health Organization guide (11). There is also a technical guide available for cold-chain transportation (12). Also, current EU regulations covering the transportation and storage have to be observed (13).

The complexity of the issue is summarized in a guide recently issued by the Irish Medicines Board (14) where it states “For many medicinal products, storage and transportation temperatures are a highly significant factor in maintaining the quality of medicinal products throughout the distribution network. The distribution chain is seldom simple and distribution systems can vary enormously. In its simplest form, the chain involves shipment direct from the manufacturer to the customer or end user but, in reality, the chain is rarely this short. In its more complex form, the distribution chain may involve a number of storage and transit locations, including airports, docks, and a variety of methods of transport, including aircraft.”

There is no doubt about the need for temperature monitoring during shipment, at least for temperature-sensitive products, e.g., products that require a cold chain, emulsions that do not tolerate freezing, suppositories, insulin injection solutions, etc. Typical situations during shipment include the following:

- A container is kept outside in freezing temperatures at the airport over night; the temperature fluctuations for a real case are shown below. An emulsion for infusion has been damaged by short-term exposure to freezing temperatures (see Fig. 6).
- Packs are transported in a closed truck on a hot day in summer.
- A container is kept at the harbor in Port Sudan for a couple of days until it clears customs.

Figure 7 shows the result of a real shipment that has been temperature monitored. The time at each temperature $>25^{\circ}\text{C}$ has been cumulated and plotted versus the temperatures. The result is quite astonishing: temperatures up to 70°C have been reached for a couple of hours, and the products shipped have been exposed to temperatures $>30^{\circ}\text{C}$ for many hours in total.

The following effects of high temperatures on heat-sensitive products are known:

- separation of emulsion systems;
- sedimentation of active ingredients in suspensions and semi-solids;
- changes in crystalline structure in fatty bases and active ingredients, resulting in changes in melting time and bioavailability; and
- increased rate of degradation leading to reduced shelf-life.

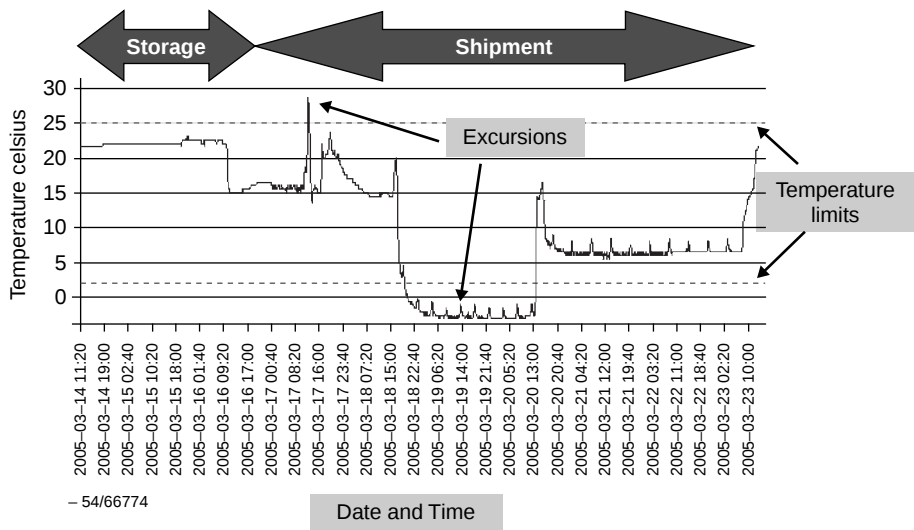


Figure 6 Temperature monitoring during shipment by airfreight for 10 days of a sterile emulsion for infusion.

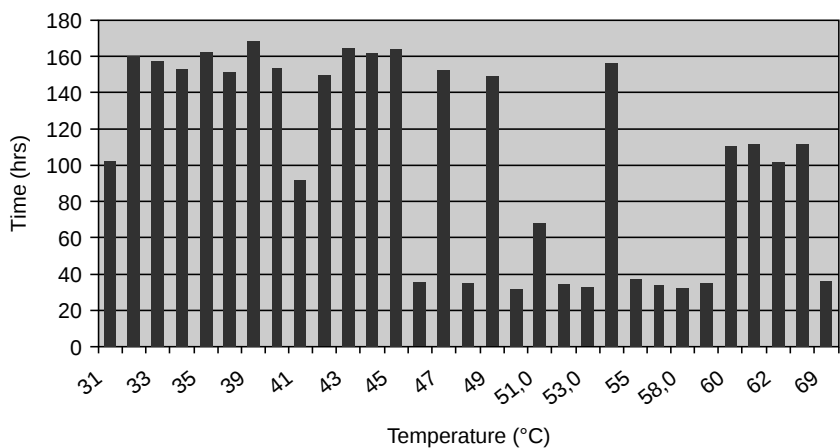


Figure 7 Temperature monitoring during shipment of tablets. Temperatures higher than 25°C are summed over time (hours).

A product defect could have the following consequences:

- harm to patients who have received the product;
- legal actions by recipients or patient groups;
- stock shortages or out-of-stock situations;
- damage to company reputation;
- cost of recovery and destruction;
- customers can switch to competitor's products;
- reduction in stock market valuation;
- penalties for non-compliance with contracts; or
- investigation by inspectors.

Enough reasons to ensure that the precious pharmaceutical products are not damaged by temperature excursions during shipment and storage.

CONCLUSIONS

Results of stress tests on the drug product are extremely helpful in evaluation of the impact of temperature excursions on the remaining shelf-life. Two aspects are essential in this respect: (i) gaining knowledge on the temperature sensitivity of the product at the development stage, and (ii) applying this knowledge on the calculation of temperature monitoring data. This enables the pharmaceutical manufacturer to estimate the remaining shelf-life, and to decide based on facts whether a product is stable until the proposed expiry date after shipment and storage. Both the unnecessary withdrawal of a stable product and the distribution of a product that does not meet the shelf-life specification can thus be avoided.

ACKNOWLEDGMENTS

I would like to thank Robert (Bob) A. Reed, USA, and Iris Allmendinger, Germany, for helpful discussions and contributions.

REFERENCES

1. Guidance for Industry: INDs for Phase 2 and Phase 3 Studies, Chemistry, Manufacturing, and Controls Information, Food and Drug Administration, Center for Drug Evaluation and Research (CDER) May 2003.
2. Waterman KC, Carella AJ, Gumkowski MJ, et al. Improved protocol and data analysis for accelerated shelf-life estimation of solid dosage forms. *Pharm Res* 2007; 24 (4): 780–90.
3. Waterman KC, MacDonald BC. Package selection for moisture protection for solid oral drug products. *J Pharm Sci* 2010; (online edition).
4. Haynes JD. Worldwide virtual temperatures for product stability testing. *J Pharm Sciences* 1971; 60: 927–9.
5. Grimm W, Schepky G. *Stabilitätsprüfung in der Pharmazie*. Aulendorf: Editio Cantor Verlag, 1980.
6. Allen PV et al. Determination of release limits: a general methodology. *Pharm Res* 1991; 8(9).
7. Research Project No. AR-4-28, Saudi Arabia (1992–1995); Research Team: Al-Shareef, A, Khalil, S. A. H., Madani, S. and Shah, A.
8. US 21 CFR 211.142 (b) (cGMP for finished pharmaceuticals); CFR 203.32 (prescription drug marketing—drug sample storage and handling requirements); CFR 205.50 (Guidelines for state licensing of wholesale prescription drug distributors—minimum requirements for the storage and handling of prescription drugs and for the establishment and maintenance of prescription drug distribution records).
9. Health Products and Food Branch Inspectorate (Canada) Guidelines for temperature control of drug products during storage and transportation, GUI-0069, January 28, 2011.
10. Good Storage and Shipping Practices USP <1079>.
11. Good distribution practices for pharmaceutical products—WHO technical report series, 2006, No 937, Annex 5, 179–203.
12. Guidance for medicinal products maintaining the quality of temperature-sensitive medicinal products through the transportation environment. *PDA Technical Report* 39 revised 2007.
13. Directive 2001/83/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to medicinal products for human use, and Guidelines on good distribution practice of medicinal products for human use (94/C 63/03).
14. Guide to control and monitoring of storage and transportation temperature conditions for medicinal products and active substances, edition IND-003 version 01, March 2006 (this guide is based on a publication by John Taylor of the UK MCA. *Pharm J* 2001; 267: 128–31).

24 | Stress testing: Frequently asked questions

Steven W. Baertschi, Karen M. Alsante, and Robert A. Reed

There are many potential problems and questions that the scientific researcher may encounter when attempting to design and carry out a stress-testing study for a pharmaceutical compound. This chapter is intended to address some of the more frequently encountered problems or questions. Since many of the questions are dealt with in more detail in other parts of the book, the reader may be referred to other chapters or publications for further information. We conclude the chapter by summarizing questions that have been received from regulatory agencies in regard to forced stress testing topics.

1. *Are stress-testing studies, the data from which may be included in the regulatory submission for a new drug entity, considered GMP studies?*

This is an important question that deserves some discussion. The stages at which stress-testing studies are carried out are generally prior to the establishment of “quality standards.” Stress-testing studies are designed to provide the groundwork for establishment of such standards. Thus, stress-testing studies should not be considered GMP studies. Instead, the focus should be on the thoroughness of the scientific investigation, soundness of design, quality of documentation, “defendability” of the conclusions, and retrievability of data.

2. *Are protocols or SOPs required for carrying out stress-testing studies?*

A survey of 20 major pharmaceutical companies indicated that 70% of the companies follow some kind of standard operating procedure and 50% require a protocol for stress-testing studies (1). There is, however, no regulatory requirement that mandates the use of protocols or SOPs.

3. *How much validation of the analytical methods used for stress-testing studies is appropriate?*

It should be remembered that stress-testing studies are investigational and the validation should demonstrate that the studies are “suitable for its intended purpose” (2). The “intended purpose” of a stress-testing method is to help understand the degradation chemistry of the drug and to provide separation and detection of as many degradation products as possible. The specificity of these methods cannot be fully validated since all the degradation products are not yet known. Validation for an investigational stress-testing method will therefore be much more limited than for official control methods. See chapter 4 for additional discussion. Nonetheless, the intent of the method does include selectivity and sensitivity, generally using the parent molecule absorbance maximum as an estimate of sensitivity for degradation products (i.e., use the relative response factor of the parent drug), and thus consideration of these validation elements are typically beneficial even at the early stage of method development.

4. *If the salt form or the physical characteristics of the drug substance change, do new stress-testing studies need to be performed?*

If either the salt form or the physical characteristics (e.g., particle size, polymorphic form, surface area, etc.) change, all solid-state stress-testing studies should be repeated, since such changes could affect degradation rates and even degradation pathways.

5. *How “hard” should a drug substance be stressed?*

This is one of the most frequently asked questions related to stress-testing studies, probably because there are no official regulatory guidances that deal with such specifics.

The primary question is whether the drug must be forced to degrade (regardless of how harsh the conditions or how long the exposure) or simply exposed to conditions of “reasonable harshness” with the understanding that if the drug does not degrade under this set of conditions then it can be regarded as “stable” to the particular stress condition. We assert that there should be realistic limits to stress-testing studies, and these limits are discussed in detail in chapter 2. Additional guidance and discussion can be found in the PhRMA “Available Guidance and Best Practices” article (3), in chapter 1, and elsewhere (4). As discussed in the PhRMA article, the target of stress testing is the “lesser of 10% degradation of the active ingredient or exposure to energy in slight excess of accelerated storage (e.g., 40°C for 6 months)” The approach discussed in the PhRMA article acknowledges that a compound may not degrade under a given stress condition after a reasonable amount of time, and that no further stressing is advised in these cases. Increasing stress conditions to force degradation (without regard to whether or not the stressing is excessive) can lead to degradation pathways that are not representative of “real world” degradation. Such degradation will cause unnecessary method development for separation of components that will never be observed under realistic conditions, for example, upon storage according to ICH guidelines (5), under light exposure (6), or under reasonable shipping or distribution excursions or patient use. A new PhRMA guidance “white paper” document (7) dealing with assessment of the formation of degradation products that may be potential genotoxic impurities also suggests that stress testing practice should focus on providing the energy “equivalent to or greater than that given to a sample in an accelerated study at 40°C over 6 months.”

6. *If a compound has aqueous solubility problems, should co-solvents be employed to facilitate dissolution for aqueous acid/base degradation studies? If so, what co-solvents are recommended?*

It is appropriate to employ a cosolvent when the solubility of the drug is limited under a given condition. The two most common cosolvents used are acetonitrile and methanol (1). It should be noted that dissolution facilitated by a cosolvent may not always increase the degradation rate, as there are many factors involved. For further discussion on this topic see “Hydrolytic” section of chapter 2.

Special attention should be given to the drug substance structure when choosing the appropriate cosolvent. One should carefully investigate the chemical composition of the drug substance and take care not to use a cosolvent that may react with it. For example, methanol and other alcohols are avoided for acidic conditions if the compound contains a carboxylic acid, ester, amide, aryl amine, or hydroxyl group. This prevents significant experimental artifact components involving reaction with methanol and other alcohols.

Also, 1 N NaOH and acetonitrile are miscible only for solutions containing 20% acetonitrile or less. Reducing the base strength to 0.1 N NaOH allows for higher levels of acetonitrile as a co-solvent. Acetonitrile is the most commonly used cosolvent (1).

7. *How do you choose which lot of a drug substance should be used in stress-testing studies? How many lots should be used for stress testing?*

Early in development, that is, preclinical, phase I or II, the answer is usually any drug you can gain access to, since material availability is often very limited during early development. However, with later phases of development, the lot of drug substance chosen for stress testing is ideally representative of the manufactured or marketed form, although this is not always possible with the solid form in the early phases of development. If the solid form changes, the solid-state stress testing should be repeated. Changes in the solid-state characteristics or polymorphic form should not require new solution studies to be performed. One lot is normally sufficient for stress-testing purposes. From the ICH Stability Guideline: “stress testing is likely to be carried out on a single batch of material (8).”

8. *Are there guidances or guidelines available for designing and carrying out stress-testing studies?*

The ICH Stability Guideline (Q1A) defines stress testing and provides a few paragraphs to describe the kinds of conditions (i.e., heat, humidity, acid/base, oxidative, and light) that should be employed during stress testing. A PhRMA working group has published an “Available Guidance and Best Practices” article on stress testing as an outcome of a workshop on the topic (3). Alsante et al. have published a useful guide for “purposeful degradation studies” (i.e., stress testing) (4) and “the role of degradant profiling in active pharmaceutical ingredients and drug products” (9). Zelesky et al. have prepared a manuscript on the “degradation and impurity analysis for pharmaceutical drug candidates” (10). Singh (11) and Silke et al. (12) have also published approaches to stress testing/forced degradation. A benchmarking study that describes common stress-testing practices of 20 major pharmaceutical companies has been published (1). For further discussion on this topic, see chapter 2.

9. *Do degradation products that arise during stress-testing studies need to be structurally characterized if they do not form at significant levels during long-term stability or accelerated stability studies in either the drug substance or product?*

There is no clear, definitive requirement in the ICH guidances or in FDA guidelines that degradation products that form only in stress-testing studies must be identified structurally. It is difficult to envision, however, how an understanding of the “intrinsic stability characteristics” of a drug compound can be developed unless some information about the structures of degradation products along with the conditions that lead to their formation is developed. This question is discussed thoroughly in chapter 2, under the section of “Intrinsic Stability: Structures of the major degradation products.” More recently, the scrutiny of degradation product structures has risen to a new level with the increased attention to potential genotoxic impurities, as described in chapter 19 of this book.

From the stress-testing benchmarking study (1), 14 companies generally identify major degradation products observed during stress testing on the drug substance even if the degradation products are not observed during formal stability studies (e.g., 25°C/60% RH, 30°C/60% RH, 40°C/75% RH). Of the 14 companies, 10 companies identify all major degradation products that form in stress testing, and two companies generally identify only those approaching ICH thresholds (i.e., the degradation products that are formed during formal stability that approach ICH thresholds).

10. *How do you choose what wavelength to monitor when performing stress-testing studies using RP-HPLC with UV detection as the analytical separation technique?*

Since both the loss of the parent drug and the increasing levels of degradation products need to be monitored, two different methods can be utilized, if desired, to analyze stressed samples, although the use of one method to monitor both has some advantages as is discussed in chapter 4. For monitoring of the parent drug, it is common to monitor the λ max of the parent. For monitoring of unknown degradation products, the goal is to maximize detectability and to minimize the differences in response factors. Monitoring at low wavelengths (e.g., 205–220 nm) is therefore recommended. With the onset of PDA detectors, it is often practiced that a broad wavelength region is monitored during method development to ascertain which wavelengths are best suited for monitoring the degradation products that form. For further discussion see chapter 4.

11. *What is a good alternate separation method to employ if RP-HPLC is the primary separation method?*

Ideally, one wants an “orthogonal” (nonoverlapping) separation technique for the alternate method. In this respect, NP-HPLC, HILIC, and RP-HPLC using different columns, pH conditions and mobile phases are a good first choice followed by CE and TLC (13). Peak purity evaluations

using photodiode array (PDA) UV analysis (to evaluate UV-homogeneity) as well as LC-MS analyses are recommended. The ICH supports this approach in suggesting, "Peak purity tests may be useful to show that the analyte chromatographic peak is not attributable to more than one component (e.g., using diode array or mass spectrometry) (6)." For more discussion of this topic, see chapters 2 and 4.

12. *How does one know whether or not all the major degradation products formed under a given stress condition are being detected with the analytical method? How do you know what the response factors of the unknown degradation product are? How should mass balance concerns be addressed during stress testing?*

These questions are critical and difficult questions, and more discussion is warranted than will be provided here. These questions are discussed in detail in chapter 2 (section XI), chapter 9 (14), and by Clarke and Norris (15). Briefly, it is nearly impossible to know whether or not all the major degradation products under a given condition are being detected until the structures of the detected products are known and the pathways of degradation can be assessed. When using UV detection, the response factors are nearly impossible to know unless purified standards are available. Mass balance concerns will always be present when using separation techniques that cannot ensure elution and resolution of all unknown products and detectors that are not universal and do not respond on the basis of mass. Whenever mass balance concerns are present, additional analysis should be performed using an orthogonal separation and/or detection scheme. The use of a detector such as a chemiluminescent nitrogen detector (which responds on the basis of the mass of nitrogen present in an eluted compound) can provide reliable response factors of unknown compounds as long as the compound contains nitrogen and the molecular formula is known. Another common detection scheme that is beneficial for poor UV/Vis chromophores is the evaporative light scattering detector (ELSD). A recently introduced alternative detector is a "charged aerosol detector" that has shown promise as a universal detector that responds to nonvolatile compounds on the basis of the amount of mass eluting off the column (16). When using UV detection, it is prudent to use PDA detection to examine the UV spectra of the parent peak and degradation products. Any degradants with UV spectra significantly different than the parent will likely have different molar absorptivity, and hence different UV response factors. The use of low wavelengths (e.g., 205–210 nm) is recommended to increase the universality of UV detection and to minimize differences in response factors.

13. *How sensitive should the analytical method be? What limit of detection is needed for routine stress-testing purposes?*

While there is not an absolute requirement for sensitivity, we advocate that a limit of detection of 0.1% of the parent molecule is appropriate for routine stress-testing purposes.

14. *At what concentration of the parent drug should acid/base aqueous stress-testing studies be carried out?*

While there are no specific requirements, most companies use a concentration between 0.1 and 1 mg/mL (1). Some companies use multiple concentrations, but most (14 out of 20 surveyed) use one concentration.

15. *Should samples be analyzed in duplicate or triplicate?*

While there are no requirements for duplicate or triplicate analysis of stress-testing samples, we recommend that samples be analyzed in duplicate, especially for studies where there are few time points or that are intended to provide results for regulatory submissions.

16. *When should stress-testing studies be performed in the development timeline for drug substance and drug product?*

This question may have several answers, as indicated by the different practices of major pharmaceutical companies. Certainly, stress testing needs to occur in order to develop and validate a stability-indicating analytical method. Because of this, some stress-testing studies need to occur at the very beginning of the development process, in the preclinical phase. Since the stability requirements for the clinical trial period are much less than what is needed for the market, stress-testing studies can be minimal in scope, with the goal of providing enough stability information to ensure the stability of the drug substance and product throughout the early clinical trials. Many companies perform additional stress-testing studies as the compound progresses through the clinical trial period to more thoroughly understand the intrinsic stability and to prepare for the needs of various formulations and of a marketed product. Determination of the structures of the major degradation products usually occurs later in the development cycle (e.g., phase II to phase III) because of the significant costs associated with isolation and structure elucidation coupled with the high fallout rate of new drugs from preclinical to the market. Furthermore, delaying these activities to later in the development programs further minimizes costs in that the major degradation products are more reflective of the final commercial product. For additional discussion of this topic see (1) and chapter 5.

17. *How much material is needed for a stress-testing study of a drug substance powder?*

The amount of material needed for a stress-testing study can vary greatly depending on the number of conditions being evaluated and the concentrations needed for the analytical evaluation. For a thorough stress-testing setup like that described in chapter 4, from 150 mg to up to 1 g may be required, although if the drug substance availability is limited, adjustments can be made to the protocols. For follow-up isolation and identification work, an additional 1–5 g may be needed to allow for generation of larger amounts of degradation products suitable for purification and structure elucidation. Alsante et al. currently request 10 mgs of drug substance for experimental purposes at the early stage of development (when there are limited quantities of material, as low as 2 mg of material if necessary) while as much as 1–5 g may be required during late stage development for thorough characterization if isolation is required for low-level degradant/impurity in the drug product especially at low API load. In terms of the amount of drug product required, early stage work requires approximately 25 tablets (solid) or 50 mL (solution) [see (4), p. 112]. Recent advances in the sensitivity of characterization techniques has helped to dramatically reduce the amounts of API or drug product needed for these studies.

18. *How do you decide which degradation products to characterize?*

While there are no official “rules,” we recommend characterization of the major degradation products formed under each stress condition that the drug degrades. A “rule-of-thumb” is to limit characterization to those products that are formed at levels greater than 10% of the total amount of degradation. Thus, if the drug degrades 10% and the individual degradation product is detected at 3% relative to the parent, this represents 30% of the total degradation and this product would be targeted for characterization. Alternatively, if the drug degrades 10% and the individual degradation product is present at 0.8% relative to the parent, this would represent only 8% of the total degradation and the product would not be targeted for characterization. See the PhRMA “Available Guidance and Best Practices” article (3), Alsante et al. (4), page 115, Alsante et al. (9), and Baertschi et al. (7), for additional references. A similar approach has recently been incorporated into a draft PhRMA white paper (7) as an algorithm to guide decisions on structure elucidation of degradation products that are discovered during stress-testing studies. The draft PhRMA paper provides a rationale for the identification thresholds proposed, describing the intended connection to ICH Q3A and Q3B identification thresholds. The proposed thresholds are shown in Chapter 2, Table 14 as a reference for the reader.

19. *How do you achieve buffered solutions across a pH range? (i.e., should one use the same buffer at ALL pH levels ...)*

Different companies use different approaches to address this problem. The use of different buffer systems that are true buffers at specific pH conditions provides for the best pH control, but the effects of the buffer on degradation rate (i.e., buffer-catalysis) can complicate the interpretation of kinetic data. The use of a single buffer system (e.g., phosphate) is possible, as long as it is recognized that not all pH ranges will be truly buffered, and the pH can therefore change upon addition of the drug substance or during the degradation process. Measuring the pH after addition of the drug substance and at the specific time points of analysis is important to ensure that the pH is maintained. For additional discussion on this topic, see Alsante et al. (1) and Waterman et al. (17).

20. *How much time and material are required to identify degradation products?*

From Alsante et al. (4), page 126: Isolation of low-level degradants can be cumbersome and time consuming. Consider a 0.1% level degradant present in a drug substance bulk lot. Based on traditional NMR experiments, 5 mg of the impurity would be needed to obtain structural confirmation. To isolate 5 mg of the impurity from the bulk, a minimum of 5 g of bulk drug substance would be needed, assuming 100% recovery. Because actual recoveries are generally closer to 50% for low-level (0.1% range) isolations, 10 g of bulk drug substance would generally be requested.

In addition to requiring significant bulk material, the timeframe to complete the isolation is considerable. If the maximum analytical load for a 4.6 mm × 150 mm column has been determined to be 5 mg, assuming the isolation will be performed using semipreparative chromatography (20 mm × 300 mm column), approximately 190 mg of sample can be loaded onto the preparative column. For a 0.1% level unknown, this translates to 190 mg of unknown injected onto the preparative column. Therefore, a total of 27 injections are required. If the assay time were estimated to be 1 hour, it would take at least 27 hour to perform the injections needed to obtain 5 mg (once again assuming 100% recovery). This timeframe does not include the time needed for method scale-up development, concentration and solubility experiments, and mass spectrometry and NMR experimentation. On the other hand, if a sample was available from stress-testing studies that contained 10% of the unknown, only 1 g of bulk would be needed and the estimated timeframe of the isolation would be drastically reduced. In the example above, 19 mg of unknown can be injected onto the preparative column (assuming the maximum analytical load does not change and resolution is retained with the higher level impurity). Therefore, only one injection would be needed to obtain the amount necessary for an NMR analysis, reducing the time to 1 hour.

21. *What degradation information is requested in a regulatory submission?*

From the PhRMA “Available Guidance and Best Practices” article on forced degradation studies (3), the following information should be supplied.

For marketing applications, current FDA and ICH guidance recommends inclusion of the results, including chromatograms of stressed samples, demonstration of the stability-indicating nature of the analytical procedures, and the degradation pathways of the drug substance in solution, solid state, and drug product. The structures of significant degradation products, that is, above the ICH identification threshold (18,19), and the associated procedures for their isolation and/or characterization also are expected to be included in the filing.

Federal regulations require more from the pharmaceutical industry than just reporting that impurities and degradation products may exist. The 1987 FDA Stability Guideline gives guidance on the procedure to follow when degradation products are detected (20):

The following information about them should be submitted when available:

- Identity and chemical structure.
- Cross-reference to any available information about biological effect and significance at the concentrations likely to be encountered.

- Cross-reference to any available information about biological effect and significance at the concentrations likely to be encountered.
- Procedure for isolation and purification.
- Mechanism of formation, including order of reaction.
- Physical and chemical properties.
- Specification and directions for testing for their presence at the levels or concentrations expected to be present.
- Indication of pharmacological action or inaction.

More specific guidance for reporting of stress testing was found in FDA draft guidance documents dealing with stability and method validation (21). According to the documents, the applicant should provide the following.

- Degradation pathways of the drug substance, alone and in drug product.
- A discussion of the possible formation of polymorphic and enantiomeric substances; the possible formation of any stereoisomers is implied.
- Data showing that neither the freshly prepared nor the degraded placebo interferes with the quantitation of the active ingredient.
- Data from stress studies of the drug substance and drug product demonstrating the specificity of the assay and analytical procedures for degradation products. These data may take the form of representative instrument output (e.g., chromatograms) and/or degradation information obtained from stress studies (e.g., results of the peak purity experiments performed on degraded samples).

22. *What degradation conditions should be performed for drug substance? Are there differences for drug product?*

Stress-testing studies of the drug substance include appropriate solution and solid-state conditions (e.g., acid/base hydrolysis, heat, humidity, oxidation, and light exposure, in accordance with regulatory guidelines) (5,20). Drug product degradation cannot be perfectly predicted from the stress-testing studies of the drug substance in the solution and solid state alone. The nonactive pharmaceutical ingredients (excipients) can also react with the drug substance or catalyze degradation reactions. Stress-testing studies of the drug product depend on the chemical composition of the drug product formulation. For drug product formulations, heat, light, and humidity are often used (3). Stress-testing experiments will vary depending on whether the formulation is a solution or solid drug product. For solid drug products, key experiments are thermal, humidity, and photostress. The most common type of interaction in solid dosage forms is between water and the drug substance; therefore, thermal/humidity challenges are critical (22). For more detailed discussion on drug substance and drug product degradation, see Alsante (4), Singh (8), and chapter 2 of this text.

23. *How should potential genotoxic degradation products be handled when conducting stress-testing studies?*

This question has become a hot topic for discussion and the approach continues to evolve as more thought and consideration is invested on this issue. Generally, a number of companies are beginning to evaluate degradation possibilities using in-silico approaches, to assess the potential for “alerting” moieties (23,24) to form with certain degradation routes. However, many companies have not yet formed a final perspective on this issue. A more comprehensive summary of this issue can be found in chapter 19 of this text.

24. *Is forced stressed testing only applicable to the active ingredient in drug products, or are there instances where forced stressed testing should also be applied to nonactive components of a drug product?*

This is a topic where the regulatory guidance is not explicitly clear. For excipients that have precedence on the market [i.e., generally recognized as safe (GRAS) or are excipients that have already been incorporated into products on the market], Brusick (25) has written a review that concludes that the inherent risk is very low from a human safety point of view. Therefore, any products that are formed from degradation of excipients alone (i.e., not a drug-excipient adduct or interaction), can be disregarded in terms of identification or safety concerns. However, there is increasing expectation that stability-indicating methods for functional excipients (e.g., lipid components of a liposomal product) also be generated. Therefore, stress testing can be useful to develop a test to evaluate the continual functionality of these important excipients.

25. *What are typical questions received from regulatory agency on the topic of forced stressed testing?*

Below are several questions that have actually been received by companies during the regulatory review process.

Question A: The stability of the drug with regard to its absolute configuration should be discussed (stereochemical stability).

Response: We described in detail a method that was able to detect diastereomers (the API had three chiral centers) of the API (method conditions and a chromatogram of the API, diastereomers, and all significant degradation products) and reported testing results for all stressed API (solution and solid state) and drug product samples—no epimerization was observed. The Agency was satisfied with this response.

Question B: Results of photostability studies in solution/suspension should be provided.

Response: Concerning forced photolytic degradation testing studies, "...testing may involve the drug substance alone and/or in simple solutions/suspensions to validate the analytical procedures" according to the International Conference on Harmonisation; Guideline for the Photostability Testing of New Drug Substances and Products; Availability [Federal Register: May 16, 1997 (Volume 62, Number 95)][Notices] [Page 27115–27122], section II, Drug Substance. Data for the drug substance stored alone (solid state) was reported in the submission. Results of ICH light storage of the oral solution in a transparent glass vial was also reported in the submission. The response was accepted.

Question C: 0.1M HCl 80°C/10 days; mass balance: what are the nature of the missing impurities (5%)?

Response: We did not investigate the unidentified impurities formed under these conditions nor did we attempt to account for the apparent lack of mass balance. According to the *International Conference on Harmonisation; Stability Testing of New Drug Substances and Products; Guideline* [Federal Register: September 22, 1994] under the heading Stress Testing (Drug Substance), elucidation of all degradation pathways may not be needed: "It is recognized that some degradation pathways can be complex and that under forcing conditions decomposition products may be observed which are unlikely to be formed under accelerated or long-term testing. This information may be useful in developing and validating suitable analytical methods, but it may not always be necessary to examine specifically for all degradation products, if it has been demonstrated that in practice these are not formed."

It is argued that the drug substance degradation results obtained in 0.1 N HCl are not relevant to the stability of the drug substance or product since (i) none of the unidentified compounds formed in 0.1 N HCl have been observed at significant levels in formal stability samples and (ii) since no mass deficit has been encountered in the formal stability trials. Moreover, mass balance *was* observed for drug substance samples stressed at 80°C in water for 10 days, 80°C in pH 6 buffer (citrate) for 7 days, under all conditions in the solid state and for the oral solution stored at 80°C for 8 days under air, results which *are* relevant to the stability of the drug substance and oral solution (pH 6, citrate). Again concerning mass balance, the guideline says "This concept (mass balance) is a useful scientific guide for evaluating data but it is not achievable in all circumstances." Such was the case here. The guideline continues: "The focus may instead be on assuring the specificity of the assay, the completeness of the investigation of routes of degradation, and the use, if necessary, of identified degradants as indicators of the extent of degradation via particular mechanisms." The data submitted do account for the degradation pathways observed in the drug substance and drug product stability studies.

Question D: Pertaining to a combination product: please conduct one time stress stability studies on a mixture of the three drug substances. These studies should include acid; base; high temperature and humidity; oxidation; and photolysis conditions.

Response: The requested experiments were conducted and the data reported. A cross product between two APIs was observed during the study, but turned out to be irrelevant to the real time stability of the drug product.

Question E: Forced degradation data do not include treatment with peroxide, which is a known contaminant of some excipients and can cause distinctive oxidative degradation. Please provide (or cite) forced degradation data for the drug substance treated with peroxides.

Response: Forced degradation data that we have provided for stressing the drug substance at 60°C for 22 hours under O₂ headspace in 3:2 water:NMP captures treatment with peroxides because based in our experience, and evidence reported in the literature¹ (reference provided below), heating NMP-water mixtures under O₂ headspace produces peroxides (NMP related hydroperoxides). The evidence for production of peroxides when the drug substance is stressed for 22 hours at 60°C under O₂ headspace in 3:2 water:NMP is further confirmed by the fact that the predominant degradation product of the drug substance under these conditions was synthesized for use as a reference marker by treatment of the drug substance freebase with hydrogen peroxide/hydrochloric acid (1).

Question F. Provide forced degradation study data (e.g., acid, base, oxidation, photostability, and heat/humidity) of the tablets and include chromatograms from the forced degradation studies.

Response: The tablets were stressed under heat/humidity (7 weeks at 60°C and 60°C/75% RH) and under light (ICH option 2; data reported in the registration document). These stress conditions adequately cover recommendations specified in guidance Q1A(R2) [section (2.2.7)] for the stability testing of drug products, which indicates that "the storage conditions and the lengths of studies should be sufficient to cover storage, shipment, and subsequent use." The tablets were not stressed in acid, base, and oxidative conditions, but the combined actives were stressed in solution under acid [(6:4) 0.1N HCl:Dimethyl sulfoxide], oxidative [(8:2) water: NMP (1-methyl-2-pyrrolidinone) under N₂, air, and O₂ headspaces], and base (0.1N NaOH).

Question G: Does the company have results of stress tests for the final product that includes chromatograms, calculations of mass balance and peak purity?

Response: Forced degradation studies have been performed on the tablets, and the tablets were stable. No new degradation products were observed when compared to those observed for the active ingredient and consequently the results were not included in the original submission. However, details of the studies are provided below. (Note, the detailed answer is not shown here, but was provided in the response to the regulatory agency.)

Question H: Refer to the validation of the analytical procedures in section 3.2.S.4.3 for the drug substance. To demonstrate the specificity of the methods for assay and related impurities for the drug substance, provide peak purity data (e.g., photo diode array analyses, selective ion recording, etc). The peak purity data should include results from the addition of known potential impurities and from forced degradation studies. In particular, demonstrate the resolution between the drug substance and the closest eluting impurity by peak purity data.

Response: Data was provided.

Question I: The following comment pertains to the validation of analytical procedure for the assay and drug-related impurities by HPLC for the drug product in section 3.2.P.5.3: The degree of the forced degradation is inadequate to demonstrate the specificity of the method. Based on the results (presented in the drug product stability section), the total impurities resulting from stressed conditions of 80°C/75%RH for 2 weeks are 2.14% and 3.29% for the (2) tablet (strengths), respectively. Provide peak purity data from forced degradation studies that achieved at least 10% degradation.

Response: In 3.2.P.8.3, we state that the objective of the study was to stress the 25 mg and 50 mg tablets to a total of 10% degradation. Current industry practice and guidance suggests that an attempt is made to attain suitable level of degradation (typically 5–20%). The tablets were exposed to 80°C/75% RH for 2 weeks. During the course of this strenuous stress study, less than 10% degradation was observed. ICH Q1A(R2) does not specifically require degradation up to a 10% level when additional degradation products are not formed under accelerated or long term storage conditions. Consequently, the forced degradation samples are sufficient to establish the specificity of the analytical method in m3.2.P.5.3.

ACKNOWLEDGMENTS

The assistance of W. Kimmer Smith and Patrick J. Jansen in reviewing this chapter is gratefully acknowledged. Also, the content of question 25 was graciously provided from the experience of employees at Glaxo-Smith-Kline, namely Maria J. Chapela, Simon R. J. Hicks, Biren K. Joshi, Sonya Kennedy-Gabb, Mark H. Kleinman, Paresh Z. Pama, and Dan W. Reynolds.

REFERENCES

1. Alsante KM, Baertschi SW, Martin L. A Stress testing benchmarking study. *Pharm Technol* 2003; 27: 60–72.
2. International Conference on Harmonisation, Guideline on Validation of Analytical Procedures, Q2A, Code of Federal Register, Vol 60, no. 40, p. 11260, March 1995.
3. Reynolds DW, Facchine KL, Mullaney JF, et al. Available guidance and best practices for conducting forced degradation studies. *Pharm Technol* 2002; 26: 48–54.
4. Alsante KM, Friedmann RC, Hatajik TD, et al. Degradation and impurity analysis for pharmaceutical drug candidates. In: Ahuja S, Scypinski S, eds. *Handbook of Modern Pharmaceutical Analysis*, chap. 4. Boston: Academic Press, 2001.

5. International Conference on Harmonisation, Stability Testing of New Drug Substances and Products, Q1A (R2), February 2003.
6. International Conference on Harmonisation, Photostability Testing of New Drug Substances and Products, Q1B, November 1996.
7. Baertschi S, Elder D, Kleinman M, et al. Strategies for addressing potentially genotoxic degradants in Active Pharmaceutical Ingredients and formulated product: PhRMA white paper proposals, PhRMA Limited Duration Key Initiative Genotoxic Impurities Team, in preparation.
8. International Conference on Harmonisation, Validation of Analytical Procedures: Methodology, Q2B, November 1996.
9. Alsante KM, Ando A, Brown R, et al. The role of degradant profiling in active pharmaceutical ingredients and drug products. *Adv Drug Delivery Rev* 2007; 59: 29–37.
10. Zelesky T, Alsante, KM, Coutant M, et al. Degradation and impurity analysis for pharmaceutical drug candidates. In: S. Ahuja, ed. *Handbook of Modern Pharmaceutical Analysis*. Volume 10. Separation Science and Technology, Elsevier, New York, October 2010.
11. Singh S, Bakshi M. Guidance on conduct of stress tests to determine inherent stability of drugs. *Pharm Technol On-Line* 2000; 24: 1–14.
12. Silke K, Muijselaar PG, Waterval J, et al. Toward a generic approach for stress testing of drug substances and drug products. *Pharm Technol* 2005; 48–66.
13. Pellett J, Lukulay P, Mao Y, et al. Orthogonal separations for reversed-phase liquid chromatography. *J Chromatogr A*. 2006; 1101: 122–35.
14. Baertschi SW. Analytical methodologies for discovering and profiling degradation-related impurities. *Trends Anal Chem* 2006; 25: 758–67.
15. Clarke HJ, Norris KJ. Sample selection for analytical method development. In: Ahuja S, Alsante KM, eds. *Handbook of Isolation and Characterization of Impurities in Pharmaceuticals*, chap 7. San Diego: Elsevier Science, 2003.
16. Dixon RW. Development and testing of a detection method for liquid chromatography based on aerosol charging *Anal Chem* 2002; 74: 2930–7.
17. Waterman KC, Adami RC, Alsante KM, et al. Hydrolysis in pharmaceutical formulations. *Pharm Dev Tech* 2002; 7: 113–46.
18. International Conference on Harmonisation, Guideline on Impurities in New Drug Substances, Q3A (R1), February 2002.
19. International Conference on Harmonisation, Guideline on Impurities in New Drug Products, Q3B (R2), June 2006.
20. US Food and Drug Administration. Drug Stability Guidelines, February 1987.
21. FDA, International Conference on Harmonization: Guidance on Q6A Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances. *Federal Register (Notices)* 65 (251), December 29, 2000: 83041–63.
22. Cartensen JT. *Drug Stability Principles and Practices*. Vol. 68. 2nd ed. New York: Marcel Dekker, 1995.
23. Muller L, Mauthe RJ, Riley CM, et al. A rationale for determining, testing and controlling specific impurities in pharmaceuticals that possess potential for genotoxicity. *Reg Toxicol Pharmacol* 2006; 44: 198–211.
24. International Conference on Harmonisation, Guideline Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use S2 (R1), March 2008.
25. Brusick DJ. A perspective on testing of existing pharmaceutical excipients for genotoxic impurities. *Regul Toxicol Pharmacol* 2009; 55: 200–4.

Index

- Absolute mass balance deficit (AMBD), 235
- Absorption, distribution, metabolism, and excretion (ADME) studies, 11–12
- Accelerated stress testing, 2, 14
- Acetals, 68
- Acetaminophen, 57
- Acid and base-catalyzed degradation, 53
- Acid and base hydrolysis, 479–480
- Acid stress, 399–402
- Acid stress-testing, 41
- Active pharmaceutical ingredient (API)
 - and excipient mixtures, 574–575
 - intermediate stability assessment, 474–475
 - photo-unstable, 218
 - stress testing, 14
- Actual degradants, 487
- Adenine nucleosides, 413–414
- Adiabatic calorimetry, 561
- Aldehydes, 65–68, 492
- Aldol reaction, 67
- Aliphatic amines, 75
- Alkenes, 199–200
- Alkyl halides, 100–103
- Allylic groups, 113
- Amadori rearrangement product (ARP), 78–80
- AMBD. *See* Absolute mass balance deficit
- AmBisome®, 431–432
- Amides, 55–59
- Amines
 - aliphatic, 75
 - aryl, 70
 - dealkylation, 75
 - heteroaromatic, 75
 - Maillard reaction, 78
 - primary, 71
 - reaction with formaldehyde and other aldehydes, 76–78
 - reaction with formulation components, 81–83
 - reaction with salts and carboxylic acids, 80–81
 - secondary, 71
 - tertiary, 72–73
- Amino acids
 - aggregation, 126
 - beta-elimination, 129
 - deamidation, 126
 - disulfide bond exchange, 127
 - hydrolysis, 127
 - isomerization, 126–127
 - oxidation, 127–128
 - photodegradation, 129
 - racemization, 126
- Amorphous freeze-dried solids
 - annealing of, 356–357
 - glass transition temperature, 353
- Amorphous solids, 271
- Ampoule calorimetry, 564–565
- Analytical methods development, 10
- Analytical testing, 228–229
- Annealing, 356–357
- Anomers, 121
- API. *See* Active pharmaceutical ingredient
- Aqueous-soluble drugs, 437–438
- Arrhenius equation, 17, 585–587
- Aryl amines, 70
- Aryl chlorides, 103
- Aryl halides, 100–103
- Atropisomerism, 512–515
- Atropisomers, 132–133
- Automation
 - biopharmaceutical formulations, 552–558
 - fully automated solid and solution state stress-testing system, 547–552
 - impact and ease of implementation, 547
 - informatics, 542
 - pH stability assessments, 546
 - sample analysis, 541–542
 - sample preparation and processing, 540–541
 - semiautomated solution state stress-testing system, 542–545
 - structure activity relationship (SAR) studies, 547
- Autoxidation
 - intrinsic stability, 23–26
 - oxidative susceptibility testing
 - azo compounds usage, 177–180
 - mechanism, 169–171
 - oxygenation requirements, 181–182
 - solution pH and temperature, 182–183
 - solvent composition, 180–181
 - unstable peroxy species, 171
- Avicel®, 295
- Aziridines, 95–96
- Base stress-testing, 41
- Basic stress, 413–415
- Batch calorimetry, 564–565
- BDE. *See* Bond dissociation energy
- Benzodiazepines, 203
- Benzyl groups, 103–108
- Beta-elimination, 129
- β -lactam, 58
- BHA. *See* Butylated hydroxyl anisole
- BHT. *See* Butylated hydroxytoluene
- Bioanalyzer®, 556–557
- Biomolecules, 207–208
- Biopharmaceutical product development
 - ICH guidelines, 378–379
 - quality by design approach, 379
 - risk assessments, 380–381
- Biophysical stability, 435–436
- Bond dissociation energy (BDE), 517–520
- Browning reaction, 66
- Butylated hydroxyl anisole (BHA), 170
- Butylated hydroxytoluene (BHT), 170
- Calorimetry, 560. *See also* Isothermal microcalorimetry (IMC)
- Cambridgesoft™, 50
- Cambridge Structural Database (CSD), 266, 504

- CAMEO. *See* Computer-assisted mechanistic evaluation of organic reactions
- Capillary electrophoresis (CE), 158
- Captured volume, 438–439
- Carbamic esters, 52–55
- Carbemazapine, 264
- Carbohydrates, 120–123
- Carbon–hydrogen bond dissociation energies, 517–520
- Carbonyl functional groups
- acetals, 68
 - aldehydes, 65–68
 - amides, 55–59
 - carbamic esters, 52–55
 - carboxylic acids, 61–65
 - esters, 52–55
 - imides, 59–61
 - ketals, 68
 - ketones, 65–68
 - lactams, 55–59
 - lactones, 52–55
- Carboxylic acids, 61–65
- Cationic lipids, 432
- CDs. *See* Cyclodextrins
- Cefaclor monohydrate, 465–468
- Cephalosporin antibiotics, 118–119
- Charged aerosol detection (CAD), 157, 246–247
- Chelating agents, 211
- ChemDraw™, 50
- Chemical degradation. *See* Degradation
- liposomes, 442
 - therapeutic monoclonal antibodies, 372–374
- Chemical instability
- light exposure, 335–336
- Chemical stability, 254
- liposomes, 426–428
 - photo sensitivity contributing factor, 327
- Chemiluminescent nitrogen detection (CLND), 247–248
- Chiral molecules, 154–155
- Cholesterol, 427–428
- Chromatographic methods
- capillary electrophoresis, 158
 - gas chromatography, 158
 - normal-phase HPLC, 157–158
 - reversed-phase HPLC
 - column selection, 155–156
 - detection, 157
 - HPLC *vs.* UHPLC, 155
 - isocratic *vs.* gradient elution, 155
 - mobile phase selection, 156–157
 - thin-layer chromatography (TLC), 158
- Chromophores, 196–197
- Clerocidin, 130–132
- CLND. *See* Chemiluminescent nitrogen detection
- Combination therapies
- literature, 449–452
 - recommended experimental approach, 453–456
 - regulatory filing strategy, 456–457
 - stress protocol, 454–455
 - survey of literature, 447–448
- Comparative stress stability studies
- analytical method, 462
 - confirmatory studies, 464
 - degradation levels, 462
 - humidity control, 462
 - kinetic models, 463–464
 - packaging materials, 462
 - representative degradation, 461–462
 - starting material
 - from multiple sources, 475–478
 - stability assessment, 474–475
 - stability-indicating analytical methods, 478–480
 - statistical design of experiments, 468–473
 - stress conditions, 461
- Comprehensive descriptors for structural and statistical analysis (CODESSA) approach, 525–526
- Computational chemistry
- applications
 - atropisomerism, 512–515
 - carbon–hydrogen bond dissociation energies, 517–520
 - conformational analysis, 512–515
 - modeling hydrolysis, 515–516
 - NMR chemical shifts, 522
 - open-shell molecules, 516–517
 - photochemistry, 523
 - quantitative structure–property relationships, 523–526
 - solvation modeling, 515
 - UV-visible spectra, 520–522
 - vibrational circular dichroism, 523
 - computer graphics, 502–503
 - databases, 503–504
 - definition, 500
 - expert system applications, 526–527
 - computer-aided synthesis, 527
 - predicting transformations, 529–530
 - reactivity and degradation, 528–529
 - toxicity predictions, 530–531
 - historical context of people and events, 501–502
 - information resources, 500–501
 - molecular mechanics, 504–505
 - practical steps, 510–511
 - quantum mechanical modeling
 - density functional theory, 507–508
 - Hartree–Fock theory, 506
 - post-Hartree–Fock methods, 508
 - semiempirical approach, 506–507
 - software, 508–510
- Computer-aided synthesis, 527
- Computer-assisted mechanistic evaluation of organic reactions (CAMEO), 528
- Computer graphics, 502–503
- Conducting photolytic stress testing, 29
- analytical testing, 228–229
 - ICH Q1B stability guideline
 - confirmatory studies, 219
 - forced degradation testing studies, 219
 - light absorption, 218
 - light exposure, 220–221
 - product development, 229–231
 - sample presentation
 - container, 224–226
 - heating effects, 227
 - humidity effects, 226–227
 - nonuniformity of illumination, 221–222
 - sample height, 223
 - sample positioning, 222–223

- Confirmatory testing studies, 219
 Conjugated ketones, 197–198
 Conjugated polyenes, 200
 Container, 224–226
 COSMO model, 515
 CPD. *See* Cyclobutane pyrimidine dimer
 Cross-polarization magic angle spinning (CP-MAS), 308
 Crystal engineering, 258
 CSD. *See* Cambridge Structural Database
 Cyclic voltammetry (CV), 187–188
 Cyclobutane pyrimidine dimer (CPD), 416
 Cyclodextrins (CDs), 211
- DaunoXome®, 431
 Dealkylation, 75
 Deamidation, 126
 Decarboxylation, 64
 Deductive estimation of risk from existing knowledge (DEREK), 486, 530
 Definitive stress testing, 12–14
 Degradants, 487
 Degradation, 11
 chemical
 liposomes, 442
 therapeutic monoclonal antibodies, 372–374
 common functional groups, 52
 common pathways, 49–52
 hydrolytic, 20–23
 oxidative
 electron-transfer mediated oxidation, 26–27
 peroxide-mediated oxidation, 26
 radical-initiated oxidation, 23–26
 photolytic, 27–29
 physicochemical, 373
 protein, 125
 during storage and shipment, 589–590
 thermolytic, 16–20
 time profiles, 584
 Degradation expert leading to pharmaceutical insight (DELPHI), 528
 Density functional theory (DFT), 507–508
 Differential calorimetry, 563
 Dimerization, 119–120
 Dioxygen. *See* Molecular oxygen
 Discoloration reaction, 66
 Doxil®, 431–432
 Drug development life cycle
 commercialization, 164–165
 drug discovery, 161–162
 line-extensions and products, 165
 preclinical/early phase, 162–164
 Drug–excipients interactions, 210–212
 Drug product stress testing, 14
 Drug substance, 2
 Drug substance stress testing, 163
 Duloxetine
 acid-catalyzed degradation pathways, 38
 hydrochloride, 37
 phthalamide, 39
 structures of, 37
 succinamide, 39
- Elaboration of reactions for organic synthesis (EROS), 527
 Electrical conductivity (EC), 246
 ELSD. *See* Evaporative light-scattering detection
 Elucidation, 36
 Emcompress®, 295
 EMEA. *See* European Medicines Agency
 Emulsion, 338–339
 Enamines, 86–87
 Encapsulants, 437–438
 Enone, 66
 Entrapment volume, 438–439
 Environmental assessment, 12
 Epimerization, 115
 Epinephrine, 100
 Epoxides, 95, 494
 EROS. *See* Elaboration of reactions for organic synthesis
 Esterification reaction, 63
 Esters, 52–55
 Ethers, 91–95
 European Medicines Agency (EMA), 484, 486
 Eutectic mixture formation, 347–349
 Evaporative light-scattering detection (ELSD), 246
 Excipients, 209–210
 attributes, 288–292
 compatibility in pharmaceuticals, 277–278
 (*see also* Solid-state excipient compatibility testing)
 parenteral drug product stress testing, 324–326
 Excipients–drug interactions, 210–212
 Expert system applications
 computer-aided synthesis, 527
 predicting transformations, 529–530
 reactivity and degradation, 528–529
 toxicity predictions, 530–531
- Fatty acids (FAs), 113–114
 Fixed dose combination (FDC), 448
 Flame ionization detector (FID), 158
 Flow calorimetry, 565
 Forced degradation testing, 40, 219
 Force field modeling, 504–505
 Formulation and packaging development, 10–11
 Freeslate Forced Degradation System, 547–552
 Freeze-dried solids
 advantages, 343–344
 amorphous *vs.* crystalline solutes, 350–351
 assessment of stability, 354–356
 excipient effects, 358–361
 glassy mixture formation, 350
 hydrate formation, 349–350
 ice–protein interactions, 361–362
 long-term stability, 362–364
 collapse effect, 364–365
 materials characterization, 351–352
 metastable glass formation, 351
 optical microscopy, 353–354
 physical chemistry of, 346–347
 physical state of drug, 357–358
 polymorphism, 349–350
 solute crystallization, 347–349
 stability, 361
 thermal analysis, 352–353

- Freeze-thaw operations, 385
 Fully automated solid and solution state stress-testing system, 547–552
 Furanoses, 120
- Gas chromatography (GC), 158
 Gas perfusion calorimetry, 565
 Gemcitabine hydrochloride
 deamination mechanism, 35
 structure of, 33
 Genotoxic impurities (GTIs). *See* Potential genotoxic impurities (PGIs)
 Glass transition temperature, 350
 Glassy mixture formation, 350
 Global Stability Testing Program, 586
 Glycine, 349
 Gordon–Taylor equation, 270
 Grotthus–Draper law, 27
 Guanine nucleosides, 414
- Hartree–Fock theory, 506
 Heat conduction calorimeters, 561–562
 Heating effects, 227
 Hemi-acetal functional group, 68
 Hemi-ketal functional group, 68
 Heteroaromatic amines, 75
 High-performance thin-layer chromatography (HPTLC), 158
 High sensitivity differential scanning calorimetry (HS-DSC), 305–306
 Humidity control, 462
 Humidity effects, 226–227
 Hydrazines, 85–86
 Hydrolytic degradation, 20–23
 Hydroxyl amine, 494
 Hydroxyl groups, 96–99
- Ice–protein interactions, 361–362
 ICH. *See* International Conference on Harmonization
 IMC. *See* Isothermal microcalorimetry
 Imides, 59–61
 Imines, 84–85
 Immunoglobulin G (IgG), 371
 Interactive generation of organic reactions (IGOR), 527
 Interfacial stress, 384–385
 International Conference on Harmonization (ICH), 1
 Intrinsic stability
 characteristics, 14
 degradation pathways, 37–39
 hydrolytic degradation, 20–23
 oxidative degradation
 electron-transfer mediated oxidation, 26–27
 peroxide-mediated oxidation, 26
 radical-initiated oxidation, 23–26
 photolytic degradation, 27–29
 rates of degradation, 29–30
 structure of major degradation products, 30–37
 thermolytic degradation, 16–20
 Investigative oxidative stress test, 27
 Investigative stress-testing tool, 28
 Iron-mediated oxidation, 434
 Isomerization reactions, 118–119
- Isothermal calorimetry, 462
 Isothermal microcalorimetry (IMC)
 adiabatic calorimetry, 561
 ampoule calorimetry, 564–565
 applications
 elevated relative humidity (RH) studies, 572
 hydrolysis reactions, 570–571
 oxidation, 571–572
 photostability, 573–574
 polymorph stability, 572–573
 primary screening, 567–569
 batch calorimetry, 564–565
 calorimeter selection, 563–564
 vs. differential calorimetry, 563
 excipient compatibility assessment, 303–305
 flow calorimetry, 565
 gas perfusion calorimetry, 565
 heat as indicator, 560
 heat conduction calorimeter, 561–562
 isothermal titration calorimetry, 566–567
 power compensation calorimeters, 560–561
 solution calorimetry, 565
 stability assessment
 API–excipient mixtures, 574–575
 formulated products, 576–578
 step-isothermal experiments, 578
 Isothermal titration calorimetry (ITC), 566–567
- Jablonski diagram, 193
- Ketals, 68
 Ketones, 65–68, 197–198
 Kinetic models, 463–464
- Laboratory Execution and Analysis (LEA)
 software, 542
- Lactams, 55–59
 Lactones, 52–55
 Lamellarity, 439–440
 Leachables, 326
 LHASA. *See* Logic and heuristics applied to synthetic analysis
 Lidocaine, 57
 Light absorption, 218
 Light exposure
 conducting photolytic stress testing, 220–221
 therapeutic monoclonal antibodies, 383–384
 Linear degradation-time profiles, 584
 Lipid peroxidation, 433
 Lipids. *See* Fatty acids (FAs)
 Liposomes
 biophysical properties, 432
 biophysical stability, 435–436
 captured volume, 438–439
 chemical degradation, 442
 chemical stability, 426–428
 effect of devices, 441
 encapsulants, 437–438
 hydrolysis, 428–432
 lamellarity, 439–440
 mechanical stress, 441
 oxidation, 432–435
 physical properties, 442
 size, 436–437
 surface charge, 440–441

- Logic and heuristics applied to synthetic analysis (LHASA), 527
- Long-term stress testing, 14
- Low-water solubility drugs
in emulsion, 338–339
functional excipients, 339
in-process stability, 339
in microemulsion, 337–338
- L-tryptophan, 76, 78
- LY334370 hydrochloride
data gathering, 145–146
experimental details, 147–151
HPLC related substances chromatograms, 151–153
preliminary studies, 146–147
solid state stress conditions, 148
solution and slurry stress conditions, 149
solutions oxidative conditions, 150
- Lyophilized drug products, 326
- mAbs. *See* Monoclonal antibodies
- Maillard reaction, 78
- Mannitol, 351
- Manufacturing parameters, 11
- Mass balance
ICH definition, 233
measurement of, 234–236
negative mass balance deficit, 242–243
in pharmaceutical analysis, 233–234
photodegraded nifedipine, 250
positive mass balance deficit, 240–242
relative response factors
charged aerosol detection, 246–247
chemiluminescent nitrogen detection, 247–248
electrical conductivity, 246
evaporative light-scattering detection, 246
mass spectral detectors, 244
in nifedipine, 248–250
and stress testing, 236
- Mass spectral (MS) detectors, 244
- MCASE. *See* Multiple computer aided structure evaluation
- Mean kinetic temperature (MKT), 587–588
- Mechanical stress
liposomes, 441
solid-state excipient compatibility testing, 295–298
therapeutic monoclonal antibodies, 384–385
- Meropenem, 83
- Metastable glass formation, 351
- METEOR, 529
- Meteor pathway prediction system (MEPPS), 529
- Microcalorimetry. *See also* Isothermal microcalorimetry (IMC)
adiabatic calorimetry, 561
heat conduction calorimeters, 561–562
power compensation calorimetry, 560–561
- MKT. *See* Mean kinetic temperature
- Modeling hydrolysis, 515–516
- Modeling photochemistry, 523
- Moisture, 462
- Molar mass balance, 234
- Molecular mechanics, 504–505
- Molecular orbital package (MOPAC), 502
- Molecular oxygen
electronic configurations, 169
oxidation, 168
- Monoclonal antibodies (mAbs)
chemical degradation
measurement techniques, 376–377
pathways, 372–374
physical denaturation
measurement techniques, 377–378
pathways, 375–376
product development tool
ICH guidelines, 378–379
quality by design approach, 379
risk assessments, 380–381
stress stability studies
freeze-thaw operations, 385
light exposure, 383–384
oxidative conditions, 382–383
pH, 381–382
physical stress, 384–385
temperature, 382
structural properties, 371–372
- MOPAC. *See* Molecular orbital package
- MultiCASE™, 486
- Multiple computer aided structure evaluation (MCASE), 530
- Nanoparticle tracking analysis (NTA), 437
- Nebulizers, 441
- Negative mass balance deficit, 242–243
- Nifedipine
photodegradation, 250
relative response factors, 248–250
- Nitriles, 68–70
- Nitroaromatic groups, 87–88
- Nitro compounds, 198–199
- Nitrogen containing functional groups
amines
aliphatic, 75
aryl, 70
heteroaromatic, 75
Maillard reaction, 78
primary, 71
reaction with formaldehyde and other aldehydes, 76–78
reaction with formulation components, 81–83
reaction with salts and carboxylic acids, 80–81
secondary, 71
tertiary, 72–73
enamines, 86–87
hydrazines, 85–86
imines, 84–85
nitriles, 68–70
nitroaromatic groups, 87–88
- NMR chemical shifts, 522
- Nonreducing sugars, 121
- Normal-phase HPLC, 157–158
- NTA. *See* Nanoparticle tracking analysis
- Nucleic acids, 123–125
- Nucleophilic oxidation, 479–480
- Olefins, 108–112
- Oligonucleotides
acid stress, 399–402
basic stress, 413–415

- Oligonucleotides (*Continued*)
 - deamination levels, 410–412
 - oxidative stress, 402–404
 - photolytic stress, 415–420
 - thermal stress, 404
- Open-shell molecules, 516–517
- Optical microscopy, 353–354
- Organic reactions accessed by computer (ORAC), 527
- Oxidation
 - amino acids, 127–128
 - iron-mediated, 434
 - isothermal microcalorimetry, 571–572
 - liposomes, 432–435
 - molecular oxygen, 168
 - nucleophilic, 479–480
 - photochemistry, 204–206
 - solid-state excipient compatibility testing, 299–301
- Oxidative degradation
 - electron-transfer mediated oxidation, 26–27
 - peroxide-mediated oxidation, 26
 - radical-initiated oxidation, 23–26
- Oxidative instability, 299, 301
- Oxidative stress
 - hydrogen peroxide, 26
 - oligonucleotides, 402–404
 - transition metals, 26
- Oxidative stress-testing, 41–42
- Oxidative susceptibility testing
 - autooxidation
 - azo compounds usage, 177–180
 - mechanism, 169–171
 - oxygenation requirements, 181–182
 - solution pH and temperature, 182–183
 - solvent composition, 180–181
 - unstable peroxy species, 171
 - goals of, 176
 - organic hydroperoxides and hydroperoxide, 171–174
 - peroxide level and temperature, 184
 - pH of cosolvent system, 185–186
 - water cosolvent system, 184–185
 - recommended conditions, 183
 - single electron transfer (SET) to dioxygen, 174–176
 - cyclic voltammetry, 187–188
 - transition metal ion catalysis, 187
 - strategy of, 188–189
- Oxone®, 26
- Oxygenation, 171
- Oxygen permeability, 302
- Packaging materials
 - comparative stress stability studies, 462
 - oxygen permeability, 302
 - water vapor transmission rates, 301
- Parenteral drug product stress testing
 - light exposure
 - chemical instability, 335–336
 - drug product administration, 330–331
 - in hospital emergency care units, 331
 - physical stability, 331–335
 - product reconstitution and administration, 331
 - low-water solubility drugs
 - in emulsion, 338–339
 - functional excipients, 339
 - in-process stability, 339
 - in microemulsion, 337–338
- photo sensitivity contributing factor
 - chemical stability, 327
 - excipients, 324–326
 - leachables, 326
 - lyophilized drug products, 326
 - photostability, 328–330
 - water-soluble drugs, 322–324
- Pathway prediction system (PPS), 529
- PDA. *See* Photodiode-array detector
- Peroxycarboximidic acid, 69
- PGIs. *See* Potential genotoxic impurities
- pH
 - of cosolvent system, 185–186
 - monoclonal antibodies, 381–382
 - stability assessments, 546
- Pharmaceutical freeze dryer, 344–346
- Pharmaceutical solids
 - in drug products
 - drug crystal forms selection, 272–276
 - excipient compatibility, 277–278
 - low level detection, 279–280
 - perspectives, 272
 - process-induced phase transformations, 278–279
 - structural diversity, 254–255
- Phenols, 99–100
- Photochemical reactions, 192–196
- Photochemistry
 - computational chemistry, 523
 - drug classification
 - alkenes, 199–200
 - aromatic and heterocyclic derivatives, 200–204
 - biomolecules, 207–208
 - chromophores, 196–197
 - inorganic compounds, 206–207
 - ketones, 197–198
 - nitro compounds, 198–199
 - oxidation process, 204–206
 - drug preparations
 - drug complex formation, 212
 - drug–excipients interactions, 210–212
 - excipients, 209–210
 - photochemical reactions, 192–196
 - physical state dependence, 208–209
- Photodiode-array detector (PDA), 541
- Photolytic degradation, 27–29
- Photolytic stress, 415–420
- Photon correlation spectroscopy, 437
- Photo sensitivity contributing factor
 - chemical stability, 327
 - excipients, 324–326
 - leachables, 326
 - lyophilized drug products, 326
 - photostability, 328–330
- Photo-unstable active pharmaceutical ingredient, 218
- Physical denaturation, 375–376
- Physical stress, 384–385
- Physicochemical degradation, 373
- Placebo blends, 279
- Polyaromatic hydrocarbons, 495
- Polymorphism, 349–350

- Polymorph screening, 11
 Polymorph stability, 572–573
 Positive mass balance deficit
 degradation products
 co-eluting with parent compound, 240
 lost from sample matrix, 239
 not detected by used detector, 238–239
 not eluted from HPLC column, 237–238
 not integrated due to poor chromatography, 240–241
 parent compound lost from sample matrix, 240
 response factors, 241–242
 Potential degradants, 487
 Potential genotoxic impurities (PGIs)
 analytical considerations, 497
 risk assessment
 combined technique studies, 486
 degradants, 487
 degradation product formation, 494–495
 in cerebro and in silico data, 488–495
 structurally alerting functional groups
 α , β unsaturated carbonyls, 493
 aldehydes, 492
 epoxides, 494
 hydroxyl amine, 494
 polyaromatic hydrocarbons, 495
 primary aromatic amine, 494
 toxicology and regulatory guidelines, 484–486
 Power compensation calorimetry, 560–561
 PPS. *See* Pathway prediction system
 Predictive oxidative stress test, 23–27
 Predictive stress testing, 12–14
 Prednisolone *tert*-butylacetate (PTBA), 256
 Primary amines, 71
 Primary aromatic amine, 494
 Primary drying, 345
 Process-induced phase transformations, 278–279
 Protein degradation, 125
 Pyranoses, 120
- Quality-by-design (QbD)
 biopharmaceutical product development, 379
 definition, 15
 Quantitative structure–property relationship (QSPR), 523–526
 Quantum mechanical modeling
 density functional theory, 507–508
 Hartree–Fock theory, 506
 post-Hartree–Fock methods, 508
 semiempirical approach, 506–507
- Racemization, 115
 Radical-initiated oxidation, 23–26, 479–480
 ReactArray semiautomated solution state
 stress-testing system, 543–545
 Reaction outcome by informatics analysis
 (ROBIA), 529
 Recommended experimental approach, 453–456
 Reducing sugars, 121
 Relative mass balance deficit (RMBD), 235
 Relative response factors (RRFs)
 charged aerosol detection, 246–247
 chemiluminescent nitrogen detection, 247–248
 electrical conductivity, 246
 evaporative light-scattering detection, 246
 mass spectral detectors, 244
 in nifedipine, 248–250
 Reversed-phase HPLC
 column selection, 155–156
 detection, 157
 HPLC *vs.* UHPLC, 155
 isocratic *vs.* gradient elution, 155
 mobile phase selection, 156–157
 Ring transformations, 116–118
 RMBD. *See* Relative mass balance deficit
 ROBIA. *See* Reaction outcome by informatics
 analysis
 Rotamer, 55
 RRFs. *See* Relative response factors
 Russell mechanism, 171
- SAR. *See* Structure–activity relationship
 Saturated fatty acids, 113
 Saturation degradation-time profiles, 584
 Secondary amines, 71
 Secondary drying, 345
 Self-consistent field (SCF), 506
 Semiautomated solution state stress-testing system,
 542–545
 Simple sugar, 120
 Solid-state excipient compatibility testing
 attributes
 active pharmaceutical ingredient, 286–288
 excipients, 288–292
 chemical instability measurement
 high sensitivity differential scanning calorimetry,
 305–306
 HPLC, 302–303
 isothermal microcalorimetry, 303–305
 design of experiments
 Box–Wilson-type fractional factorial design, 293
 Plackett–Burman saturated factorial design,
 293–294
 kinetics and decay extrapolation, 310–315
 parameters, 286–287
 physical form stability and assessment, 306–310
 stress conditions
 mechanical stress, 295–298
 oxidation, 299–301
 packaging materials, 301–302
 thermal conditions, 295
 water, 298–299
 Solid-state form screening
 amorphous forms, 268–271
 cocrystals, 262–264
 drug product design, 258–260
 polymorphs, 264–268
 salt formation, 260–261
 solvates, 264–268
 Solid-state reaction kinetics, 257–258
 Solid-state reactivity, 255–258
 Solid-state stress testing, 40–41
 Solute crystallization, 347–349
 Solution calorimetry, 565
 Solution photostability stressing, 480
 Solvation modeling, 515
 SPARC, 525
 SpectraMax®, 556

- Stability-related stress testing
 - absorption, distribution, metabolism, and excretion (ADME) studies, 11–12
 - analytical methods development, 10
 - environmental assessment, 12
 - formulation and packaging development, 10–11
 - manufacturing parameters, 11
 - polymorph screening, 11
 - processing parameters, 11
 - safety issues, 11
 - salt selection, 11
 - toxicological issues, 11
- Statistical design of experiments, 468–473
- Storage and shipment
 - Arrhenius equation, 585–587
 - Arrhenius plot, 587
 - assumptions, 589
 - degradation, 589–590
 - limitations, 590
 - mean kinetic temperature, 587–588
 - regulatory requirements, 591–592
- Stress
 - acid, 399–402
 - basic, 413–415
 - interfacial, 384–385
 - mechanical, 295–298, 384–385, 441
 - oxidative, 402–404
 - photolytic, 415–420
 - physical, 384–385
 - thermal, 402
- Stress protocol, 454–455
- Stress test screen
 - data gathering, 142
 - methods of analysis
 - chiral molecules, 154–155
 - chromatographic methods, 155–159
 - specific *vs.* generic methods, 153
 - validation methods, 154
 - preliminary studies, 143
 - sample preparation
 - buffer and solution preparation, 145
 - slurry sample, 144
 - solid-state samples, 144
 - solution samples, 144
 - standards, 144–145
 - suspension sample, 144
- Structure–activity relationship (SAR), 161–162, 547
- Sublimation, 343
- Sugars, 120–123
- Sulfonamides, 88–90
- Sulfonyl functional groups
 - alkyl halides, 100–103
 - allylic groups, 113
 - aziridines, 95–96
 - benzyl groups, 103–108
 - epoxides, 95
 - ethers, 91–95
 - fatty acids, 113–114
 - hydroxyl groups, 96–99
 - olefins, 108–112
 - phenols, 99–100
 - sulfonamides, 88–90
 - sulfonylureas, 90
 - thioethers, 91–95
 - thiols, 90–91
- Sulfonylureas, 90
- Surface charge, 440–441
- “Take off” degradation-time profiles, 584
- Termination reaction, 171
- Tertiary amines, 72–73
- Tertiary hydroxyls, 98
- Theoretical degradants, 487
- Therapeutic monoclonal antibodies. *See* Monoclonal antibodies (mAbs)
- Thermal analysis, 352–353
- Thermal stress, 402
- Thermolytic degradation, 16–20
- Thin-layer chromatography (TLC), 158
- Thioamides, 55
- Thioethers, 91–95
- Thiols, 90–91
- Threshold of toxicological concern (TTC), 486
- Thymine nucleosides, 413
- Topochemical reactions, 256
- Total attenuated reflectance IR spectroscopy, 308–309
- Toxicity prediction by computer-assisted technology (TOPCAT), 530
- Toxtree™, 486, 491
- Transition metals, 479–480
- Triamcinolone acetonide, 68–69
- Triethylammonium acetate (TEAA), 395
- TTC. *See* Threshold of toxicological concern
- Ultra high pressure liquid chromatography (UHPLC), 155
- Ultraviolet absorbance, 238
- UV-visible spectra, 520–522
- Vibrational circular dichroism (VCD), 523
- Vitamin E, 113
- Vitrification hypothesis, 362
- Vogel–Tammann–Fulcher (VTF) equation, 355
- Water-soluble drugs, 322–324
- Water substitute hypothesis, 362
- ZENETH, 530

PHARMACEUTICAL STRESS TESTING

PREDICTING DRUG DEGRADATION

SECOND EDITION

ABOUT THE BOOK

The second edition of *Pharmaceutical Stress Testing* approaches the topic of stress testing with the underlying theme that stress testing is predictive in nature, multi-dimensional (analytical, organic, physical, spectroscopic), and an integral part of the drug development process.

Whilst thoroughly updating existing topics, the second edition also extends coverage into oligonucleotides and proteins, and offers increased information covering formulated products and diverse dosage forms. Further updates include; discussion of chemical stability and the physical aspects of stress testing; the concept of 'Quality by Design'; the connection between stress testing and 'real world' stability through topics related to kinetics, predictive models, and actual conditions experienced by drugs and drug products during shipping and distribution. The role of new technologies for designing, interpreting, or carrying out stress testing studies is also discussed.

Key features include:

- A renowned Editorial team and international contributions from major drug companies, reflecting a wealth of experience
- 10 new chapters, including Stress Testing and its relationship to the assessment of potential genotoxic degradants, combination drug therapies, proteins, oligonucleotides, physical changes, and alternate dosage forms such as liposomal formulations
- Updated methodologies for predicting drug stability and degradation pathways
- Best practice models to follow

ABOUT THE EDITORS

Steven W. Baertschi is a Research Fellow, Eli Lilly and Company. Dr Baertschi's research interests include impurity isolation/purification and structure elucidation, mechanisms of drug degradation reactive intermediates and drug-excipient interactions. Dr Baertschi has organized or chaired several scientific symposia on forced drug degradation, photostability and impurity investigations and has published widely on the topics. Dr Baertschi received his degree in chemistry from David Lipscomb University, Nashville, Tennessee and received his PhD in organic chemistry from Vanderbilt University, Nashville, Tennessee.

Karen M. Alsante is a Research Fellow, Pfizer PharmaTherapeutics Pharmaceutical Sciences in Groton, Connecticut. Dr Alsante's experience has included leading drug degradation/impurity isolation, solid state characterization as well as early analytical research activities starting in partnership with Discovery and progressing through development to Phase 2 clinical trials. Dr. Alsante organized and chaired several scientific conferences on forced drug degradation, photostability and oxidation and has published extensively on these topics. Dr Alsante received a BA with honors in chemistry from Holy Cross College in 1989 and her PhD in organic chemistry from Duke University in 1994.

Robert A. Reed, is Vice President, CMC and Technological Operations for Celsion Corporation overseeing the CMC, QA and Technical Operations functions. Dr Reed most recently was Vice President, Pharmaceutical Operations at XenoPort, Inc., and has over 20 years of experience across Celsion Corp., XenoPort, Inc, Merck & Company, Inc., and The Liposome Company, Inc., with extensive scientific and regulatory experience in the design and development of pharmaceutical products. Dr Reed holds a Ph.D. in Analytical Chemistry from The University of North Carolina at Chapel Hill and was the recipient of a 3 year NIH Postdoctoral Individual Award at Princeton University.

Drugs and the Pharmaceutical Sciences series has been widely recognized as a leading source of information for the pharmaceutical science industry for more than 30 years. Over 200 volumes covering a broad range of topics within pharmaceutical science – from drug discovery, development, delivery, manufacturing, engineering and pharmaceutical statistics, through to brand management, marketing and packaging – make this a must-read resource for scientists and industry professionals. Led by Dr James Swarbrick, Series Editor and an international Editorial Board the volumes are available in both print and online formats. For more information please see www.informahealthcarebooks.com

Series Executive Editor: James Swarbrick.

Series Advisory Board: Larry L. Augsburger, Harry G. Brittain, Jennifer B. Dressman, Robert Gurny, Anthony J. Hickey, Jeffrey A. Hughes, Joseph W. Polli, Kinam Park, Yuichi Sugiyama, Elizabeth M. Topp, Geoffrey T. Tucker, Peter York.

Cover photograph of Core Module 3 system used with permission from Freeslate, Inc.

informa
healthcare

Telephone House, 69-77 Paul Street, London EC2A 4LQ, UK
52 Vanderbilt Avenue, New York, NY 10017, USA

www.informahealthcare.com
www.informahealthcarebooks.com

ISBN 978-143980179-6

